Mechanisms Underlying Gemcitabine Resistance in Pancreatic Cancer and Sensitisation by the iMiD™ Lenalidomide

ROSEMARY A. FRYER1, BLAKE BARLETT2, CHRISTINE GALUSTIAN1 and ANGUS G. DALGLEISH1

1Department of Oncology, Division of Cellular and Molecular Medicine, St. George’s University of London, U.K.; 2Celgene Corp., Summit, NJ, U.S.A.

Abstract. Gemcitabine is currently the leading therapeutic for pancreatic cancer treatment, despite growing resistance. Studying the mechanisms that underlie gemcitabine resistance and discovery of agents that increase the tumour sensitivity to gemcitabine, is therefore desirable. The thalidomide analogue lenalidomide has been approved for use in multiple myeloma in combination with dexamethasone. Although it is primarily immunomodulatory, it also has direct effects on tumours. We investigated the sensitivity of three pancreatic cell lines PANC-1, MIA-PaCa-2 and BxPC-3 to gemcitabine. We observed that PANC-1 cells display most resistance to gemcitabine and MIA-PaCa-2 are most sensitive. Western blot analysis revealed that PANC-1 exhibits high phosphorylated extracellular signal-regulated kinase (pERK) expression, whereas MIA-PaCa-2 displays low expression. Combining gemcitabine and lenalidomide reduced the IC50 of gemcitabine up to 40% (p<0.05). Western blot analysis showed lenalidomide significantly reduced pERK expression in all cell lines (p<0.05). It was hypothesised that gemcitabine sensitivity could be increased through combination with a pERK-reducing agent. The mitogen-activated kinase (MEK) specific inhibitor U0126 was used on PANC-1 cells to restore gemcitabine sensitivity. U0126 significantly increased cell killing by gemcitabine from 30% to 60% (p<0.001). Sensitive MIA-PaCa-2 cells were transfected with a constitutively active MEK mutant to reduce gemcitabine sensitivity. Transfection resulted in a significant reduction in cell killing by gemcitabine from 54-16% (p<0.05). These results provide evidence that ERK activity underlies sensitivity to gemcitabine and that addition of an agent that reduces this activity, such as lenalidomide, enhances gemcitabine efficacy. In conclusion, these results provide an understanding of gemcitabine resistance and could be used to predict successful combination therapies.

Current statistics place pancreatic adenocarcinoma as the fifth leading cause of cancer related death in the world. This type of cancer is responsible for a death rate almost identical to its incidence rate (1). Patients diagnosed with this disease can expect a 1-year survival rate of approximately 10%, with only 4.6% surviving beyond 5 years with treatment (2). The high mortality observed with pancreatic cancer is undoubtedly attributed to inadequate treatment. Currently the nucleoside analogue gemcitabine is the leading therapeutic for pancreatic cancer because of its ability to improve quality of life and overall survival (3). However, due to the growing number of patients exhibiting resistance to this drug (4), there is renewed interest in developing new treatment regimes.

The mechanisms by which gemcitabine resistance occurs are still under heavy investigation. Through in depth research, it has been shown that aberrant activity in signalling pathways that modulate the cell cycle and apoptosis in pancreatic cancer, plus disruption of gemcitabine metabolism to its active form, correlate with gemcitabine resistance (5-8). The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway is known to be important in the development of pancreatic cancer and is also highly dysregulated in the disease (9, 10). The MAPK/ERK pathway is a pro-survival pathway has been previously implicated in chemoresistance (11-14). Activation of this pathway influences angiogenesis, cellular proliferation, apoptosis, and survival.

Different approaches, such as the development of new drugs, have been adopted in order to find more effective treatments. One approach gaining popularity is combinatorial treatment. The rationale to this approach is that the administration of a second drug may be able to increase the efficacy of the first drug, such as gemcitabine. This may be due to the second agent acting directly to increase cell killing or by interfering with

Correspondence to: R.A. Fryer, Department of Oncology, Division of Cellular and Molecular Medicine, 2nd Floor Jenner Wing, Rm 2.50, St. George’s University of London, London, SW17 0RE, United Kingdom. Tel: +44 (0)208725 5307, Fax: +44 (0)208725 0158, e-mail: rosie.fryer@googlemail.com

Key Words: Pancreatic cancer, gemcitabine, resistance, ERK, lenalidomide.
mechanisms that induce resistance to gemcitabine. To date, gemcitabine combined with either capcitabine or erlotinib has been shown to be superior to gemcitabine alone in clinical trials (15-18). However, the drawback with combining therapies is that there is an increased prevalence of side-effects and the aforementioned combinations are particularly toxic. Thus, it is desirable to find agents with a limited toxicity profile that enhance gemcitabine efficacy.

Lenalidomide is an analogue of thalidomide which has been approved for use in multiple myeloma in combination with dexamethasone, and although it primarily acts as an immunomodulator, it has also been reported to be antiproliferative, antiangiogenic and pro-apoptotic (19-21). Lenalidomide is known to increase the sensitivity of myeloma cells toward other chemotherapeutic agents (22). In light of this, a single case study of metastatic pancreatic cancer has emerged where lenalidomide used in combination with gemcitabine resulted in a better outcome than in any study using gemcitabine alone (23). The precise mechanism by which gemcitabine action might be potenitated by lenalidomide is yet unknown.

It is hypothesised that pancreatic cancer cells rely on the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) pathway for proliferation and that aberrant activation of this pathway could in fact confer gemcitabine resistance. The aim of this study was therefore to establish the molecular signature of gemcitabine in pancreatic cancer cell lines in vitro and to determine causes of resistance. In addition, the mechanism by which lenalidomide enhances gemcitabine efficacy was investigated.

**Materials and Methods**

**Cell culture.** The pancreatic cancer cell lines PANC-1, MIA-PaCa-2 and BxPC-3 were obtained from the European Cell and Culture Collection (ECACC, Salisbury, UK). PANC-1 and MIA-PaCa-2 cells were maintained in Dulbecco’s modified Eagle’s Medium (DMEM; Sigma Aldrich, Poole, UK) supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine and 2 mM penicillin/streptomycin. BxPC-3 cells were maintained in RPMI-1640 medium supplemented with 20% FBS, 4 mM L-glutamine and 2 mM penicillin/streptomycin. Cell lines were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

**Reagents.** Gemcitabine (gemcitabine hydrochloride) was obtained from St. George’s Hospital Pharmacy, sourced from Eli Lilly (Surrey, UK). This was reconstituted in 0.9% (w/v) saline solution to a 200 mM stock solution and stored at room temperature. Lenalidomide was obtained from the Celgene Corporation (New Jersey, USA), dissolved in 100% dimethyl sulfoxide (DMSO) to produce stock solutions of 10 mM that were stored at -20°C.

**Assessment of gemcitabine efficacy in pancreatic cancer cell lines.** To study the effect of gemcitabine on cell growth of PANC-1 and MIA-PaCa-2 cells, the CellTiter-Blue® Cell Viability assay (Promega, Southampton, UK) was used, and for BxPC-3 cells the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. For both assays, cells were harvested with Accutase (TCS CellWorks, Buckingham, UK) and resit in 96-well plates in their respective media at a density of 0.5-2×10⁵ cells/well. Gemcitabine was added to the wells so that the final volume per well was 100 μl across the plate. Plates were incubated for 48 h at 37°C. MTT (5 mg/ml, dissolved in phosphate buffered saline) was added to each well containing BxPC-3 cells, and CellTiter-Blue® reagent was added to PANC-1 and MIA-PaCa-2 cells. Plates were returned to the incubator for 4 h. MTT plates were spun at 500 × g for 5 min, the medium aspirated off and 100 μl DMSO added. Plates were gently agitated and then read at 550 nm on a spectrophotometer. Cells treated with CellTiter-Blue® reagent were also read at 550 nm. To determine the concentration at which cell viability was reduced by 50% (IC₅₀), the following equation was used:

\[
E_P = E_C - \frac{E_{\text{max}} + C^n}{IC_{50}^n + C^n}
\]

Where, \(E_P\)=predicted effect, \(E_C\)=control effect, \(E_{\text{max}}\)=max effect, \(C\)=concentration of drug, and \(n\)=sigmoid-fit factor

Modulation of gemcitabine efficacy by a fixed dose of lenalidomide. PANC-1 cells were plated at a density of 5×10³ cells/well in DMEM, in 96-well plates. Cells were treated with Gemcitabine (0.001-10 mM) in addition to medium, 0.1 μM or 1 μM lenalidomide. Cell viability was tested after 48 h at 37°C, using the CellTiter-Blue® cell viability assay as previously described.

**Immunoblotting analysis.** Total cellular protein from PANC-1, MIA-PaCa-2 and BxPC-3 cells was extracted using Cell Lysis Buffer (Cell Signaling Technology, Dancers, USA), according to the manufacturer’s protocol. Lysates were used with a human phospho-MAPK Proteome Profiler™ array (R&D Systems, Abingdon, UK). This was used as a screen in order to create a panel of ‘proteins of interest’, as per the manufacturer’s instructions. Array results were quantified using Western blot analysis. Lysates were resolved by Tris-Bis gel electrophoresis using a 4-12% gradient gel. Proteins were transferred to a nitrocellulose membrane and blocked with 5% (w/v) non-fat milk in TBST [0.1% (v/v) Tween-20 in tris-buffered saline (100 mM Tris hydrochloride, 150 mM sodium chloride, pH 7.0)]. Membranes were then probed with antibodies against phospho-ERK1/2 (pERK) and ERK1/2 (tERK). All antibodies were obtained from Cell Signaling Technologies and used at a dilution of 1:2000. Membranes were then probed with antibodies against GAPDH (1:2000) and β-actin (1:2000). Membranes were developed using X-ray film. Analysis was performed by pixel densitometry using Adobe Photoshop CS3.
Inhibition of MAPK(ERK1/2) signalling in gemcitabine-resistant PANC-1 cells. PANC-1 cells were harvested and plated at 1×10^5 cells/well in 6-well plates, in the presence of 3 μM of the MEK-specific inhibitor U0126. After 24 h, cells were harvested and re-cultured in basal medium or medium with 100 μM gemcitabine for a further 48 h at 37˚C. Cell numbers were then assessed by cell counting with trypan blue.

Transient transfection of gemcitabine-sensitive MIA-PaCa-2 cells. The human haemagglutinin (HA)-tagged constitutively active MEK-R4F plasmid and the dominant negative mutant MEK-8E were kind gifts from Dr Natalie Ahn (University of Colorado). MIA-PaCa-2 cells were plated at a density of 2×10^5 cells/well in a 24-well plate in DMEM supplemented with 10% FBS and 2 mM L-glutamine and incubated overnight at 37˚C. Cells were treated with Lipofectamine 2000 reagent according to the manufacturer’s protocol (Invitrogen, Paisley, UK) and plasmids were introduced and cells incubated for a further 18-24h at 37˚C. Transfection success was assessed by flow cytometric analysis of HA-tag using an anti-HA-tag-FITC antibody (Abcam, Cambridge, UK). Plates were then incubated for a further 48 h at 37˚C before cell count by trypan blue staining.

Statistical analysis. Values are presented as mean±standard deviation. Statistical analysis of results was by repeated measures one-way ANOVA with Bonferroni post-hoc test and p<0.05 was considered significant.

Results

Gemcitabine sensitivity differs between pancreatic cancer cell lines, but lenalidomide does not induce cell death. PANC-1, MIA-PaCa-2 and BxPC-3 cells were cultured with a range of gemcitabine doses and then analysed using cell viability assays. The reason two different assays were used is that BxPC-3 cells appeared not to be able to metabolise the CellTiter-Blue® reagent, so it was necessary to revert to the MTT assay. Results show a dose-dependent relationship that differs between the cell lines (Figure 1). Determination of gemcitabine IC_{50} values by using the E_{max} equation reveals that the order of gemcitabine sensitivity is PANC-1<BxPC-3<MIA-PaCa-2 (Table I). Used alone, the agent lenalidomide was found not to cause cell death of any of the cell lines over the range of concentrations used (Figure 2).

Level of MAPK/ERK pathway activation correlates with sensitivity to gemcitabine. Whole cell lysates from untreated cell lines were analysed by Proteome Profiler™ arrays (R&D Systems) and then quantified by Western blot analysis. Results showed that sensitivity of each cell line inversely correlated with its level of pERK expression (Figure 3). MIA-PaCa-2 cells were most sensitive to gemcitabine and displayed a low basal expression of pERK. In contrast, PANC-1 cells were the least sensitive to gemcitabine and displayed a high basal expression of pERK. BxPC-3 cells were mildly sensitive to gemcitabine although the level of pERK expression did not reflect this.

Treatment of PANC-1 cells with the MEK-specific inhibitor U0126 restores sensitivity to gemcitabine. To examine the effect of reducing MAPK/ERK activity on the sensitivity of the gemcitabine-resistant PANC-1 cells, the MEK-specific inhibitor U0126 (Promega) was used. U0126 was titrated in order to find a concentration that reduced pERK expression and caused minimal cell death. PANC-1 cells were incubated with 3 μM U0126 for 24 h at 37˚C. Cells were then washed and fresh media containing 100 μM gemcitabine was added to cells and incubated for a further 48 h at 37˚C. Cell counting with trypan blue showed that 81.0±3.6% cells were alive after treatment with U0126 alone, 69.7±1.5% cells were alive after gemcitabine treatment alone and 39.0±3.6% alive.

Table I. Table of IC_{50} values of gemcitabine determined for PANC-1, MIA-PaCa-2 and BxPC-3 cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC_{50} (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANC-1</td>
<td>300±33</td>
</tr>
<tr>
<td>MIA-PaCa-2</td>
<td>61±3</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>128±16</td>
</tr>
</tbody>
</table>
after treated with both U0126 and gemcitabine. There was a significant difference between each of these values when compared to control cells (p<0.001). Further to this, the observed reduction in the percentage of live cells was significantly greater after treatment with both agents than either one alone (p<0.001) (Figure 4).

Transient transfection of MIA-PaCa-2 cells with a constitutively active MEK mutant reduces sensitivity to gemcitabine. In parallel to the U0126 treatment of PANC-1 cells, the effect of increasing MAPK/ERK activity was investigated in MIA-PaCa-2 cells. This was done by transiently transfecting MIA-PaCa-2 cells with the constitutively active MEK1-R4F mutant. These cells designated MIA-PaCa-2R4F, expressed higher basal levels of pERK compared to the non-transfected MIA-PaCa-2 cells. Wild-type and mutant MIA-PaCa-2 cells were treated with 100 μM gemcitabine or media alone. Cell number was assessed using trypan blue staining. The numbers of MIA-PaCa-2wt and MIA-PaCa-2R4F were 16.0±2.6 and 30.7±2.8×10⁵ cells/well. The difference in these values were found to be significant (p<0.05) demonstrating a reduction in sensitivity to gemcitabine in cells containing the MEK-R4F mutant (Figure 5).

Lenalidomide reduces pERK expression and enhances gemcitabine efficacy in pancreatic cancer cell lines. The immunomodulatory agent lenalidomide was tested in combination with gemcitabine in order to investigate potential enhancement. Initial studies into the effect of lenalidomide on pancreatic cancer cells using MTT and CellTiter-Blue® assays showed that lenalidomide does not induce cell death over a range of concentrations. The ability of low-dose lenalidomide to modulate gemcitabine efficacy in PANC-1 was assessed. Concomitant treatment of PANC-1 cells with a range of doses of gemcitabine and 1 μM lenalidomide resulted in an IC₅₀ significantly lower than treatment with either agent alone (40% reduction, p<0.05, Figure 6A). In parallel studies, a single dose of gemcitabine (100 μM) was combined with a single dose of lenalidomide (1 μM). The observed percentage cell kill was then determined for the treatment of PANC-1 cells with gemcitabine alone.
lenalidomide alone and concomitant treatment with both agents. It was found that the combination of gemcitabine and lenalidomide resulted in significantly greater cell kill than that induced with either agent alone \((p<0.01)\). The observed kill for the combination was greater than that of the expected kill (determined by the sum of the kill induced by both agents individually), although this was not found to be significant. Western blot analysis of whole cell lysates from cells treated with a non-toxic dose of lenalidomide \((1 \mu M)\) revealed that expression levels of pERK in PANC-1 cells were significantly reduced in comparison to untreated cells \((p<0.05)\), from 85% to 32% expression.

**Discussion**

In this paper, we present data that indicates gemcitabine resistance may be related to an overexpression of pERK and therefore overactivation of the MAPK/ERK signalling pathway. We also show that by reducing the activity of this pathway with the immunomodulatory agent lenalidomide, sensitivity to gemcitabine is restored. The involvement of the ERK signalling pathway has been previously reported in other studies \((24, 25)\), although this is the first time that it has been shown that the lenalidomide is able to reduce expression of pERK, and therefore ERK activation, thereby restoring gemcitabine sensitivity in pancreatic cancer cells \textit{in vitro}. Data have shown that the sensitivity of PANC-1, MIA-PaCa-2 and BxPC-3 pancreatic cancer cell lines to gemcitabine differs greatly. PANC-1 cells proved to be the most resistant and possess the highest level of ERK activation as shown by pERK expression. In comparison, MIA-PaCa-2 cells showed the greatest sensitivity to gemcitabine and possessed the lower ERK activation compared to PANC-1 cells. BxPC-3 cells showed intermediate sensitivity to gemcitabine but also exhibited low activity of the ERK pathway. The only difference in phenotype between the three cell lines used was that PANC-1 and MIA-PaCa-2 are \(KRAS\) mutant and BxPC-3 are \(KRAS\) wild-type. This could explain the low ERK activity seen in the BxPC-3 cells. To investigate whether this relationship between ERK activation and gemcitabine sensitivity truly exists, the MEK-specific irreversible inhibitor U0126 was used to treat the gemcitabine-resistant PANC-1 cells. Results showed that by pre-treating the cells with a low dose of U0126 that caused minimal cell death, sensitivity to gemcitabine was restored. This was confirmed by Western blot analysis, which showed a reduction in pERK expression induced by U0126. In contrast, sensitive MIA-PaCa-2 cells were transfected with a MEK mutant that led to constitutive activation of the ERK signalling pathway. Treatment of the MEK mutant cells revealed significantly less killing by gemcitabine than in the wild-type cells. As a logical last step to demonstrate the existence of this relationship between ERK activation and gemcitabine, the novel agent lenalidomide, known to reduce ERK activity, was used in conjunction with gemcitabine on PANC-1 cells. Concomitant treatment of cells with gemcitabine and lenalidomide resulted in more killing than with either agent alone. Together these results indicate that through addition of an ERK-inhibiting agent, the efficacy of gemcitabine in
pancreatic cancer cell lines is increased, and suggest that the overactivation of ERK signalling has a role to play in the resistance to gemcitabine.

But how does reduction in the activity of the ERK pathway lead to increased gemcitabine efficacy? The most common mutation seen in pancreatic cancer is in KRAS (~90%), an upstream effector of the MEK/ERK pathway. The mutation in KRAS produces a constitutively active protein (26), which leads to an overactivation of the aforementioned pathway. It has been postulated that because of the occurrence of KRAS mutations and the subsequent activation of the MEK/ERK pathway, that this is an ideal target for development of new therapies (27). The activation of ERK leads to the regulation of a variety of processes including cell proliferation, apoptosis and survival. The reliance of pancreatic cancer cells on the ERK signalling pathway has been demonstrated through experiments involving inhibitors of MEK, and transfection of dominant negative mitogen-activated protein kinase kinase kinase 1 (MEKK1) mutants into cell lines (28, 29).

Downstream, ERK is responsible for the regulation of the BCL-2 family of proteins, involved in apoptosis (30).
A common hallmark of cancer is an imbalance in apoptotic protein expression, favouring survival. An overexpression of BCL-2 related proteins via ERK signalling has been observed in pancreatic cancer and is associated with resistance to the induction of cell death by various mechanisms (31, 32).

Included in this family are the anti-apoptotic proteins, myeloid cell leukaemia sequence 1 (MCL-1) and BCL-xL. In pancreatic cancer, MCL-1 is highly up-regulated and therefore antiapoptotic mechanisms and cell survival are favoured (33, 34). This up-regulation could in fact be due to the overactivation of ERK, due to expression being directly modulated by this pathway.

MCL-1 overexpression has been shown to confer resistance to gemcitabine in many types of cancer, including pancreatic cancer, and knockdown by targeted siRNA restores efficacy of gemcitabine (35). In addition, it has also been shown that there is an increase in mRNA levels of MCL-1 and BCL-xL after gemcitabine treatment (36), which could in fact potentiate the resistance. Treatment of pancreatic cancer cells with antisense oligonucleotides against BCL-xL increase sensitivity and induction of apoptosis by gemcitabine in vitro (37).

Preclinical studies of inhibitors of MCL-1 combined with gemcitabine have been shown to synergistically increase the cytotoxic and apoptotic action of gemcitabine in pancreatic cancer cells, both in vitro and in vivo (38). This observation has also been made for bladder cancer and squamous head and neck carcinoma (39, 40).

The observation made in this study that pre-treatment of pancreatic cancer cells with the MEK-specific inhibitor U0126 resulted in significantly greater killing by gemcitabine confirms that the ERK signalling pathway is important in gemcitabine resistance. The reduction in ERK activation leads to a reduction in MCL-1 expression, an effect that is demonstrated in other types of cancer (41, 42). This in turn eliminates gemcitabine resistance mechanisms and restores the cytotoxic effect of gemcitabine, as demonstrated in the current study. The efficacy of CCI-1040, a MEK1/2-specific inhibitor, has been tested in phase I/II trials for colon cancer but did not advance to phase III due to poor pharmacokinetics (43).

From the results of this study, it was hypothesised that an agent that can reduce the activity of the ERK pathway should therefore enhance the action of gemcitabine in pancreatic cancer cells. The immunomodulatory agent lenalidomide is approved for use in multiple myeloma and its effects are now well documented. Although lenalidomide has been shown to induce apoptosis in myeloma cells (44-46), this was not observed in the current study with pancreatic cancer cells. Despite not inducing apoptosis, lenalidomide did reduce pERK expression in PANC-1, MIA-PaCa-2 and BxPC-3 cells. This effect has been demonstrated previously in an in vitro study with myeloma-derived cell lines (47). Using two combination

Figure 5. Effect of transient transfection of MIA-PaCa-2 cells with a constitutively active MEK mutant. A: Cell counting by trypan blue staining showed that in non-transfected MIA-PaCa-2 cells, 100 μM gemcitabine significantly reduced the number of live cells after 48 h. In mutant MIA-PaCa-2R4F cells, 100 μM gemcitabine did not significantly reduce the number of live cells. The difference in the number of live cells in both cell types after gemcitabine treatment was also significantly different. B: Transfection success was confirmed by the detection of HA-tag by Western blot analysis. Blotting also confirmed the increase in pERK expression in the mutant MIA-PaCa-2R4F cells compared to the wild-type MIA-PaCa-2 cells.
models, it was confirmed that lenalidomide enhanced the efficacy of gemcitabine in gemcitabine-resistant PANC-1 cells. These cells also displayed high pERK expression which was attenuated after treatment with lenalidomide but not gemcitabine. This effect of lenalidomide on pancreatic cancer cells has not previously been seen in any study.

**Conclusion**

The growing resistance to gemcitabine in pancreatic patients is a problem that is in urgent need of resolving. In this study, it has been demonstrated that by deciphering the precise mechanisms by which this resistance occurs, it is possible to create combination regimes using novel agents that are superior to gemcitabine alone.

**Acknowledgements**

The Authors would like to thank the Ralph Bates Pancreatic Fund for their generous funding towards this research. Additional support was provided by the Cancer Vaccine Institute, to whom we are also very grateful.
References


3755


