Abstract. Despite incremental advances in the diagnosis and treatment for pancreatic cancer (PC), the 5-year survival rate remains <5%. Novel therapies to increase survival and quality of life for PC patients are desperately needed. Epigenetic therapeutic agents such as histone deacetylase inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMTi) have demonstrated therapeutic benefits in human cancer. We assessed the efficacy of these epigenetic therapeutic agents as potential therapies for PC using \textit{in vitro} and \textit{in vivo} models. Treatment with HDACi [suberoylanilide hydroxamic acid (SAHA)] and DNMTi [5-AZA-2' deoxycytidine (5-AZA-dc)] decreased cell proliferation in MiaPaCa2 cells, and SAHA treatment, with or without 5-AZA-dc, resulted in higher cell death and lower DNA synthesis compared to 5-AZA-dc alone and controls (DMSO). Further, combination treatment with SAHA and 5-AZA-dc significantly increased expression of p21\textsuperscript{WAF1}, leading to G1 arrest. Treatment with epigenetic agents delayed tumour growth \textit{in vivo}, but did not decrease growth of established pancreatic tumours. In conclusion, these data demonstrate a potential role for epigenetic modifier drugs for the management of PC, specifically in the chemoprevention of PC, in combination with other chemotherapeutic agents.

Introduction

Pancreatic cancer (PC) is the fourth most common cause of cancer death in Western societies with a 5-year survival of <5% (1-6). PC often presents asymptomatically, and as a consequence is advanced in the majority of cases at diagnosis (5,7). Surgical resection currently offers the only option for long-term survival, however, only 20% of patients are suitable for surgical intervention (8,9). Current chemotherapeutic and radiation treatments have also met with limited success (5,9). Novel diagnostic and therapeutic strategies are urgently needed to improve outcomes for this disease.

Epigenetic therapeutic agents, such as histone deacetylase inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMTi), have demonstrated efficacy in the treatment of cutaneous T-cell lymphoma (CTCL) (10-12) and myelodysplastic syndrome (MDS) (13,14). Preclinical studies of epigenetic modulating drugs in other cancers have also demonstrated antitumour activity with tolerable toxicity, suggesting a potential role for these drugs in cancer treatment (15-19). HDACi and DNMTi are thought to act by regulating gene expression and remodelling the chromosome structure, which may lead to cell cycle arrest and cell death (20,21).

HDACi induce accumulation of acetylated histones, resulting in the relaxation of chromatin structure to promote access of transcriptional machinery (22). Induction of p21\textsuperscript{WAF1} expression after treatment with HDACi is common and appears to play a major role in arresting the growth of transformed cells (23-26). DNMTi reduce genomic DNA methylation by binding to DNA methyltransferases (DNMT) after being incorporated into newly synthesized DNA (16,27). DNA methylation, a major epigenetic mechanism of gene regulation,
is usually associated with gene silencing (28). Treatment with DNMTi reverses aberrant DNA methylation and thus reactivates the transcription of many genes, including putative tumour suppressor genes (16,28,29). The re-expression of tumour suppressor genes is thought to contribute, at least in part, to the DNMTi anticaner effect (29,30). In acute myeloid leukemia cells, DNMTi alter cell cycle progression, reduce cell proliferation and induce apoptosis (16). Treatment combining DNMTi with HDACi results in synergistic cell death, which may reflect re-expression of silenced genes as well as the potentiation of cell death through acetylation of non-histone proteins (10,31).

Previous studies have identified several genes with tumour suppressor properties that are epigenetically regulated in PC (32-35), including the regulation of mucin expression [Muc1 (36), Muc2 (37) and Muc4 (38)], which is associated with carcinogenesis and tumour invasion (36-39). The mechanism of epigenetic alterations in PC is poorly understood (40). Treatment of PC cells with HDACi induces cell death and enhances the apoptotic effects of gemcitabine (24). In the gemcitabine-resistant PC cell line, Panc1, treatment with suberoylanilide hydroxamic acid (SAHA) restores sensitivity to gemcitabine (24). Treatment with 5-AZA-deoxycytidine (5-AZA-dc) restores the expression of Bnip3 and induces hypoxia-mediated cell death (35). Together, these data suggest potential for epigenetic modulating drugs in the treatment and management of PC.

Materials and methods

Cell culture. MiaPaCa2 cells were cultured in DMEM supplemented with 10% FBS and 2.5% horse serum according to American Type Culture Collection (ATCC) protocols. Human pancreatic ductal epithelial (HPDE) cells, a gift from Dr Ming-Sound Tsao, were used as a normal control and cultured in keratinocyte serum-free medium (KSF) supplemented with 50 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor (41). These cell lines were maintained in a humidified atmosphere of 5% CO2 at 37°C.

5-AZA-dc and SAHA treatment. MiaPaCa2 cells were plated at 1.5x10^5 cells/well in 6-well plates for protein/nucleic acid harvest; or at 3.9x10^5 cells in T25 flasks for flow cytometry analysis (Day 0). The cells were treated as follows: i) treated on Day 1 with 300 µM of 5-AZA-dc (Sigma-Aldrich) and harvested on Day 6; ii) treated on Days 1-3 and Day 5 with 5 µM of SAHA (Cayman Chemical) and harvested on Day 6; iii) treated on Day 1 with 5-AZA-dc, Day 2, 3 and 5 with SAHA and harvested on Day 6. Untreated MiaPaCa2 and HPDE cells were used as controls. During treatment, the media was changed daily, and cells were washed twice with cold PBS prior to nucleic acid and/or protein extraction.

MTS assay. An MTT assay was performed using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay according to the manufacturer's protocol (Promega Corporation). MiaPaCa2 and HPDE cells were plated at a density of 0.5x10^5 cells/well in 96-well plates to measure cell proliferation. Cells were treated on Days 1-5 with SAHA (Cayman Chemical) at a concentration of 1, 3 or 5 µM. Untreated MiaPaCa2 and HPDE cells were included in each plate as controls. Each treatment group was plated in triplicate and repeated at least three times. Media was changed daily during treatment. An additional untreated plate was prepared as a baseline. Absorbance was measured at 490 nm.

Brdu and PI staining. MiaPaCa2 cells were pulsed with bromodeoxyuridine (Brdu) (Sigma-Aldrich) for 1 h prior to harvesting. Cells were harvested and fixed in 70% ethanol overnight at 4°C before Brdu and propidium iodide (PI) (Sigma-Aldrich) staining. The next day, ethanol was removed and cells resuspended in PBS/1% Tween-20 (PBST) followed by DNA denaturation using 1.5 M HCl for 20 min. The cells were in PBST (3X), resuspended in 100 µl PBST, followed by the addition of 5 µl of 250 µg/ml FITC-anti-Brdu (MAB3262F; Chemicon) for 1 h at 37°C in the dark. After incubation, cells were washed with 1 ml cold PBST and resuspended in 470 µl PBST. PI (5 µl of 1 mg/ml) and RNase A (25 µl of 10 mg/ml) (Sigma-Aldrich) were added and mixed gently by pipetting. The cells were incubated between 1-4 h in the dark at room temperature. Prior to running on the BD FACSCalibur™ or BD FACSCanto™ flow cytometer (BD Biosciences), cells were syringed gently to avoid cell clumping. Flow cytometry data were analysed using either BD CellQuest™ (BD Biosciences), or FlowJo 8.7.3 or 8.8.2 (Tree Star, Inc.).

Protein extraction. Cell lysis and protein extraction from cell lines were performed on ice. Media was removed and cell monolayers washed twice with cold PBS. Lysis buffer (50 µl) containing protease inhibitors (0.5% deoxycholate, 150 mM NaCl, 1% sodium pyrophosphate, 50 mM Tris pH 8.0, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 200 µM sodium orthovanadate; Sigma-Aldrich) was added to each well of a 6-well plate. The cells were scraped and transferred to microcentrifuge tubes, vortexed and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was collected and 2-5 µl of the supernatant was used for protein quantification. The remaining supernatant was stored at -80°C. Protein quantification was performed using the Bio-Rad Protein Assay kit according to the manufacturer's instructions.

Western blot analysis. Following normalization for protein concentration, SDS sample buffer was added and lysates denatured at 70°C for 10 min, protein was separated using 4-12% Bis-Tris NuPAGE® precast gels (Invitrogen Life Technologies) and transferred to polyvinylidene fluoride (PVDF) membranes according to the manufacturer's protocols. Non-specific binding was blocked in 10% (w/v) skim milk powder in TBS/Tween-20 [10 mM Tris pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20; TBST]. Membranes were incubated with primary antibodies diluted in TBS/BSA solution (10 mM Tris pH 7.4, 150 mM NaCl, 5% w/v BSA, 0.02% azide). Table I contains the list of primary antibodies and their incubation conditions. Membranes were washed in TBST for 30 min, then incubated for 1 h at room temperature with HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1:2,000; Amersham Pharmacia Biotech) in 5% (w/v) skim milk supernatant was collected and 2-5 µl of the supernatant was used for protein quantitation. The remaining supernatant was stored at -80°C. Protein quantification was performed using the Bio-Rad Protein Assay kit according to the manufacturer's instructions.

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Table I. Primary antibodies for western blot analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Incubation</th>
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<tbody>
<tr>
<td>β-actin (AC-15; A5441)</td>
<td>1:40,000</td>
<td>Sigma-Aldrich</td>
<td>1 h RT</td>
</tr>
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<td>1:500</td>
<td>Santa Cruz. Biotechnology, Inc.</td>
<td>O/N 4°C</td>
</tr>
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<td>E (He12; SC-247)</td>
<td>1:100</td>
<td>Novocastra</td>
<td>O/N 4°C</td>
</tr>
<tr>
<td>A (C19; SC-596)</td>
<td>1:2,000</td>
<td>Cell Signaling Technology, Inc.</td>
<td>O/N 4°C</td>
</tr>
<tr>
<td>D1 (DCS-6)</td>
<td></td>
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<tr>
<td>B1 (V152; 4135)</td>
<td></td>
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</tr>
<tr>
<td>p21waf1 (610234)</td>
<td>1:1,000</td>
<td>BD Biosciences</td>
<td>O/N 4°C</td>
</tr>
</tbody>
</table>

**RT**, room temperature; O/N, overnight.

powder in TBST. Membranes were washed for 30 min in TBST before proteins were visualized using the Enhanced Chemiluminescence (ECL) detection system (Perkin Elmer) on X-ray film (Fujifilm Medical Systems USA). Cell cycle marker (cyclin E, A, D1, B1 and p21WAF1) protein levels were determined relative to β-actin by densitometry using ImageJ software [National Institutes of Health (NIH)] and normalized to DMSO-treated control samples.

In vivo study of the efficacy of epigenetic therapeutic agents in a xenograft model of PC. Ethics approval was obtained from Garvan Institute and St. Vincent’s Hospital Animal Ethics Committee to examine the effect of epigenetic therapeutic agents in a mouse xenograft model of PC (Protocol no. 07/06).

MiaPaCa2 cells are injected subcutaneously into female athymic nude mice (BALB/c nu/nu) (6–8 weeks old, mean weight of 16 g). Prior to injection, MiaPaCa2 cells were harvested, counted and diluted to the appropriate concentration in cold media. For each mouse, 1.0x106 MiaPaCa2 cells were diluted in 1:1 mixture of cold media and BD Matrigel™ Basement Membrane Matrix (BD Biosciences) to a total volume of 100 µl and kept on ice until injection.

The mice were divided into three treatment groups: i) vehicle (control) (n=5), ii) SAHA alone (n=5); and iii) 5-aza-dc and SAHA (n=5). 5-aza-dc was administered in a single dose at a concentration of 0.25 mg/kg, followed by daily intraperitoneal administration of SAHA for 21 days at a dose of 50 mg/kg (in a maximum volume of 0.1 ml). Treatment began 1 week after the injection of MiaPaCa2 cells to examine the ability of the drugs to prevent tumour growth (Prevention Studies); or once a solid tumour was present to test the ability of the drugs to reduce tumour growth (Therapeutic Studies). The mice were euthanased upon cessation of drug treatments and tumour weight was measured. The pancreas, abdominal cavity, mesentery, spleen and liver were assessed for the presence of metastases. Tumour size was measured, and tumour volume was calculated using the formula 1/2 length x breadth x width (42). The in vivo experiments were performed in duplicate, with tumour measurements from each experiment combined for statistical analysis.

Statistical analysis. All in vitro experiments were carried out at least in triplicate, and the in vivo studies in duplicate. Mean, standard deviation and Student’s t-test were calculated using Microsoft Excel. Statistical analyses of univariate analysis of variance (ANOVA), Tukey’s post hoc tests and calculation of 95% confidence interval were performed using SPSS16.0 (SPSS Inc.) and/or R v.2.10.1 (R Foundation for Statistical Computing).

**Results**

Treatment with SAHA and 5-aza-dc decreases cell proliferation in MiaPaCa2 cells. Treatment with SAHA alone, or in combination with 5-aza-dc, resulted in higher cell death and lower DNA synthesis (Fig. 1A) compared to 5-aza-dc alone or DMSO-treated cells. In MiaPaCa2 cells, treatment with SAHA was more effective in inducing cell death than treatment with 5-aza-dc (p=0.002 and p=0.008 respectively), while SAHA treatment alone was not significantly different to combination therapy (p=0.019; Fig. 1A). The dramatic effect of SAHA may potentially be due to a global effect of chromatin remodelling in regulation of cellular function, as defective histone dynamics during S phase, DNA repair and mitosis result in cell cycle arrest and cell death (43).

DNA synthesis levels, as measured by BrdU incorporation, were significantly reduced upon treatment with SAHA, either alone (p=0.001) or in combination with 5-aza-dc (p=0.005), while treatment with 5-aza-dc alone had no significant effect compared to DMSO-treated cells (Fig. 1A). These data correlate with higher levels of p21WAF1 observed in SAHA and SAHA/5-aza-dc combination-treated samples relative to control and 5-aza-dc-treated cells (Fig. 1B), which is consistent with findings in other cancer types (44).

Western blot analysis of cell cycle markers cyclin E1, B, A, D1 and p21WAF1 revealed that MiaPaCa2 cells treated with 5-aza-dc alone expressed higher cyclin E protein compared to control (DMSO) cells (Fig. 1B). Cells treated with SAHA, alone or in combination with 5-aza-dc, expressed higher levels of cyclin E1 and significantly higher p21WAF1 protein levels that could be contributing to G1/S-phase cell cycle arrest (45).

MiaPaCa2 cells are more sensitive than HPDE cells to treatment with SAHA. Previous studies have reported that normal cells are more tolerant to epigenetic drug therapy compared to cancer cells (46-49). We investigated this premise using the ‘normal’ pancreatic ductal cell line, HPDE, and MiaPaCa2 cells.

Increasing SAHA concentration significantly decreased cell proliferation in both MiaPaCa2 (p<0.0001) and HPDE cells (p<0.0001; Fig. 1C). However, the cell lines demonstrated different cell proliferation rates in response to drug treatment, suggesting that MiaPaCa2 cells are more sensitive to epigenetic modifying agents than HPDE cells (p=0.034).

Treatment with SAHA and/or 5-aza-dc increases expression of p21WAF1. Induction of p21WAF1 expression upon treatment with epigenetic drugs reportedly induces cell cycle arrest in some cancer types, including MiaPaCa2 cells. The induction of p21WAF1 expression in HPDE and MiaPaCa2 cells after treatment with SAHA, with or without 5-aza-dc (Fig. 1D),
was associated with decreased proliferation (Fig. 1C), suggesting that the induction of p21<sup>WAF1</sup> expression following treatment with epigenetic drugs may contribute to decreased cell proliferation.

The efficacy of 5-AZA-dc and SAHA in a pancreatic xenograft model

**Prevention studies.** Using a subcutaneous xenograft model of PC we investigated the efficacy of pharmacological epigenetic modulation in the prevention of tumour growth. Treatment with SAHA and/or 5-AZA-dc significantly increased the tumour lag period compared to control (p=0.001 and p<0.001 respectively; Fig. 2A), while the lag period between the two treatment groups (5-AZA-dc/SAHA and SAHA alone) was similar (p=0.882; Fig. 2A). The lag period was defined as the time required for the tumour volume to reach 100 mm<sup>3</sup> (indicated by the shaded area in Fig. 2A). Tumour growth rate measured after the lag cut-off point (100 mm<sup>3</sup>), was significantly different between groups (p=0.001) (Fig. 2A), indicating an effect following treatment. However, overall tumour growth rates between the two treatment groups (5-AZA-dc/SAHA and SAHA alone) were similar. No significant difference was observed in the mean tumour weights between all groups upon completion of treatment (p=0.994). This suggests that after the lag period, tumours in the treated groups grew at an increased rate when compared to the control group.

**Therapeutic studies.** As PC is usually diagnosed at an advanced stage, we investigated the ability of epigenetic therapies to reduce tumour growth using an established subcutaneous xenograft model of PC. Similar tumour growth rates were observed
between treatment groups and control (p=1.000; Fig. 2B), with no significant difference in tumour weight observed (p=0.448). These results indicate that in this model, treatment with SaHa, alone or in combination with 5-aZa-dc, was not effective in the treatment of established pancreatic tumours \textit{in vivo}.

**Discussion**

Epigenetic therapies have shown promising antitumourigenic effects in some malignancies (15-19,50). These include enzyme inhibitors, specifically DNMTi and HDACi, which induce epigenetic modifications. In this study, we demonstrated that treatment of PC cells with HDACi (SAHA) in combination with DNMTi (5-AZA-dc) decreased cell proliferation and induced cell death. This may be mediated through upregulation of p21\(^{WAF1}\), and is associated with cell cycle arrest, apoptosis and decreased cell proliferation (23-26,44). This study also demonstrated that MiaPaCa2 cells were more sensitive to epigenetic drugs compared to the ‘normal’ HPDE pancreatic cells.
Treatment with SAHA induced significant cell death and reduced DNA synthesis, potentially as a result of cell cycle arrest. Dysregulated histone modification, which can be promoted by HDACi, may lead to aberrant chromatin remodelling during DNA replication and repair, and mitosis (43,51,52). Treatment with SAHA also induces a more open chromatin structure, increasing the susceptibility of DNA to damage (53,54). These events correlate with cell cycle arrest leading to cell death (43,51,52). Our study of MiaPaCa2 cells demonstrate that treatment with SAHA (HDACi) was more effective than treatment with 5-AZA-dc (DNMTi) alone. Consequently, aberrant chromatin modification following treatment with HDACi, such as SAHA, may play an important role in an anticancer effect. While the effect of HDACi in chromatin remodelling would also apply to normal cells, these are likely to be more resistant to epigenetic treatment (46-49). Therefore, this mechanism does not fully explain the anticancer effects of SAHA in MiaPaCa2 cells, or the higher tolerance of HPDE cells to epigenetic treatment.

Our data show that decreased cell proliferation and induction of cell death following treatment with SAHA and 5-AZA-dc may be mediated via upregulation of p21\(^{WAF1}\). p21\(^{WAF1}\) is tightly regulated by p53, however, as the MiaPaCa2 cell line used in this study is p53 defective, p21\(^{WAF1}\) must be regulated by pathways independent of p53 [26,55,reviewed in (56)]. Previous studies have indicated that treatment with epigenetic drugs is likely to increase the expression of p21\(^{WAF1}\) by regulating the chromatin structure and increasing the acetylation of histone 3 on the p21\(^{WAF1}\) promoter (12,23,26,57,58). More recently, Vijayaraghavalu et al demonstrated that sequential treatment of resistant breast cancer cells with 5-AZA-dc and doxorubicin induces a highly synergistic effect and caused the resistant cells to undergo G2/M cell cycle arrest, which was due to upregulation of p21\(^{WAF1}\) expression. Induction of p21\(^{WAF1}\) was correlated with depletion of DNA methyltransferase 1 (DNMT1), which promotes DNA methylation, suggesting that p21\(^{WAF1}\) may be a methylation-suppressed gene in specific cell types (59).

A further explanation for the reduction of MiaPaCa2 cell proliferation observed in our study may be via the process of autophagy. Recent studies provide a strong link between HDAC inhibition and cell death by the process of autophagy (60,61). In particular, Robert et al (61) showed that using valproic acid, a class I and II HDAC inhibitor, triggers Sae2 (CtIP in human) degradation by promoting autophagy that affects the DNA damage sensitivity of hist1 and rpdr3 mutants. While beyond the scope of this study, further experiments are necessary to address the difference in cellular response in cancer and normal cells after treatment with epigenetic drugs, such as SAHA, particularly in the way these cells regulate chromatin remodelling (61).

Since the effectiveness of epigenetic therapy is cell and context-dependent (10,22,45,62), the anticancer activities \textit{in vitro}, may not be translated into \textit{in vivo} settings. Thus the efficacy of epigenetic drugs \textit{in vivo} was assessed using a subcutaneous xenograft model of PC. The prevention studies demonstrated that treatment with SAHA, alone or in combination with 5-AZA-dc, delayed tumour progression during the early stage (lag period) of tumour development. However, the therapeutic studies demonstrated that treatment with SAHA, with or without 5-AZA-dc, did not reduce tumour growth rate nor tumour weight, indicating that treatment with epigenetic drugs alone is unlikely to be effective for the treatment of established pancreatic tumours. These data suggest that treatment with epigenetic drugs during early pancreatic carcinogenesis may provide an opportunity for the use of combination treatment with other chemotherapeutic drugs, thereby increasing the susceptibility of tumour cells to cytotoxic agents, with many studies demonstrating the synergistic effect of epigenetic drugs with existing therapeutic agents (10,19,53,63-65). In particular, SAHA increases the sensitivity of PC cells to the chemotherapeutic agent gemcitabine (24). This may be particularly useful in an adjuvant setting, where a systemic adjuvant approach for resected pancreatic adenocarcinoma is the most effective means for improving overall survival (66-69). This approach has been investigated by Mohammed et al (70), who demonstrated the chemopreventative efficacy of the EGFR inhibitor, gefitinib, in delaying the progression of pancreatic intraepithelial neoplasia (PanIN) lesions to pancreatic adenocarcinoma, while not showing efficacy in advanced disease.

The exact mechanisms of the anticancer activity of combination treatments have yet to be fully elucidated, but may include regulation of chromatic structures and the induction of pro-apoptotic genes (19,24,64,65,71). Our data show that the epigenetic agents modulate the cell cycle and inhibit cell growth, however, one important question arises. How effective are combination treatments likely to be if many anticancer agents need an efficient cell cycle to exert their effect? Venturelli et al recently showed that the epigenetic agent 5-AZA-cytidine sensitises cancer cells to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) by: i) inhibiting protein biosynthesis of tumour-protecting factors, thus enabling TRAIL-induced apoptosis; and ii) reversing the malignancy-associated methylation phenotype. The ability of 5-AZA-cytidine to inhibit protein biosynthesis was associated with the ability of the drug to be incorporated into cellular RNA and disrupt cellular protein biosynthesis (72). This study suggested that epigenetic drugs could exert their anticancer mechanisms via non-epigenetic modes of action, which may provide a more complete picture of the anticancer activities of combination treatments with epigenetic agents. Further, in a recent study by Shakya et al, 5-AZA-dc was administered in a mouse model of aggressive stromal-rich pancreatic adenocarcinoma, and demonstrate that 5-AZA-dc significantly reduced DNA methylation and slowed progression of pancreatic adenocarcinoma. 5-AZA-dc upregulated interferon-inducible genes (e.g., STAT1), and that treatment with 5-AZA-dc and interferon γ had an antiproliferative effect (73). These studies support the rationale for future studies combining epigenetic agents with other anticancer agents, as well as with cytokines and immunotherapy.

In conclusion, this study illustrated that treatment with epigenetic agents decreased cell proliferation and induced cell death in PC cells, while \textit{in vivo} studies demonstrated a delay in tumour progression following treatment. These data suggest that epigenetic therapy has the potential to delay early pancreatic carcinogenesis, and may have potential application in an adjuvant setting for the management of resected PC.
References


