

Targeting pancreatic cancer using a combination of gemcitabine with the omega-3 polyunsaturated fatty acid emulsion, Lipidem™.

Jonathan Haqq^{1,2}, Lynne M. Howells^{1*}, Giuseppe Garcea², Ashley R. Dennison².

Author affiliations:

1) University of Leicester, Dept. Cancer Studies, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester, UK, LE2 7LX.

2) Dept Hepatobiliary Surgery, Leicester General Hospital, Gwendolen Road, Leicester, UK, LE5 4PW.

***Corresponding author:** Lynne M. Howells. University of Leicester, Dept. Cancer Studies, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester, UK, LE2 7LX. Email: lh28@le.ac.uk

Tel: +44116 2522271

Abbreviations:

DHA – docosahexanoic acid; DMEM – Dulbecco’s modified eagle’s medium; DMSO – dimethyl sulfoxide; ECM – extracellular matrix; ELISA – enzyme linked immunosorbent assay; EPA – eicosapentanoic acid; FACS – fluorescence activated cell sorting; FBS – foetal bovine serum; FITC – fluorescein isothiocyanate; IMDM – Iscove’s modified Dulbecco’s medium; PBS – phosphate buffered saline; PDGF – platelet-derived growth factor; PDGFR – platelet-derived growth factor receptor; PSC – pancreatic stellate cell; PUFA – polyunsaturated fatty acid; SDS – sodium dodecyl sulfate; SHP-2 – Src-homology phosphatase-2.

Keywords:

Fishoil; polyunsaturated fatty acids; pancreatic cancer; pancreatic stellate cells; platelet-derived growth factor.

Abstract

Scope: Pancreatic cancer remains a disease of poor prognosis, with alternate strategies being sought to improve therapeutic efficacy. Omega-3 fatty acids have shown clinical benefit, and mechanisms of action are under investigation.

Methods/results: Proliferation assays, flow cytometry, invasion assays, ELISA and western blotting were used to investigate efficacy of omega-3 fatty acids alone and in combination with gemcitabine. The docosahexanoic acid (DHA)/eicosapentanoic acid (EPA) combination, Lipidem™, in combination with gemcitabine inhibited growth in pancreatic cancer and pancreatic stellate cell (PSC) lines, with PSCs exhibiting greatest sensitivity to this combination. Invasion of pancreatic cancer cells and PSCs in a 3D spheroid model, was inhibited by combination of gemcitabine with Lipidem™. PSCs were required for cancer cell invasion in an organotypic co-culture model, with invasive capacity reduced by Lipidem™ alone. Platelet derived growth factor (PDGF) is a key cytokine in pro-proliferative and invasion signalling, and thus a critical regulator of interactions between pancreatic cancer cells and adjacent stroma. Platelet-derived growth factor (PDGF-BB) secretion was completely inhibited by the combination of Lipidem™ with gemcitabine in cancer cells and PSCs.

Conclusion: Lipidem™ in combination with gemcitabine, has anti-proliferative and anti-invasive efficacy *in vitro*, with pancreatic stellate cells exhibiting the greatest sensitivity to this combination.

Introduction

The omega-3 polyunsaturated fatty acids (PUFAs) docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA), which occur in oily fish such as salmon, mackerel, and tuna, are considered to be very healthy constituents of the human diet. Increasingly they are also employed as medical treatments to alleviate specific conditions. One condition in which potential for beneficial effects is being investigated, is pancreatic cancer. Pancreatic cancer represents 3% of all cancer cases in the UK, and offers a dismal prognosis with a 5- and 10-year survival rate of 3% and 1% respectively. There has been little improvement in survival rates over the last 40 years, with incidence rates closely mirrored by mortality rates [1].

Whilst surgery provides a potentially curative option for pancreatic cancer [2], only approximately 15% of cases are suitable for surgical resection, with the majority of patients presenting with advanced disease or overt metastases at the time of diagnosis [3].

Gemcitabine has long been the mainstay of chemotherapy for pancreatic cancer, with pre-operative chemotherapy improving survival in patients with locally advanced disease [4], and in the palliative setting for those patients with poor performance status [5]. Recent studies have also suggested that combination of therapeutics with gemcitabine, such as nab-paclitaxel [6], confers a survival advantage when compared to gemcitabine monotherapy in patients with metastatic pancreatic cancer, but may come with a price of increased toxicities.

The incidence of pancreatic cancer has been found to be inversely correlated with intake of PUFAs, including DHA and EPA [7]. Furthermore, administration of EPA and DHA postoperatively, is able to improve pancreas and liver function in patients undergoing surgery for pancreatic/gastrointestinal cancers [8]. However, the greatest potential for clinical utility of PUFAs arises within the palliative setting. A growing number of studies suggest that incorporation of PUFA-enriched supplements into nutritional support regimens for pancreatic cancer patients, may be of clinical benefit without adding to side effect burdens. Cancer-related cachexia is a major cause of morbidity in pancreatic cancer patients, which may be alleviated by PUFAs such as EPA, resulting in net weight gain and improved quality of life [9,10]. Importantly, the safety of high dose omega-3 fatty acids is well established, and so may provide an ideal low toxicity adjunct for standard care gemcitabine-based regimens in pancreatic cancer. Establishing mechanisms by which PUFAs contribute to potential anti-cancer efficacy of chemotherapy over and above cachexia alleviation, have more recently been investigated in patients with advanced pancreatic cancer, by the Dept. Hepatobiliary Surgery, Leicester, UK. Here, patients with locally advanced or metastatic disease underwent

standard of care gemcitabine treatment, immediately followed by an intravenous omega-3 fatty acid-rich emulsion (Lipidem™) [11]. Significant decreases in serum platelet-derived growth factor (PDGF) were observed for a proportion of patients in this study, with these responders exhibiting improved overall survival compared to the non-responders. Despite this observation clinically, there is still a paucity of data establishing how such mechanism-based observations may contribute to the combined efficacy of PUFAs and gemcitabine. This is essential if this combination is to gain further credence for clinical utility in the palliative setting. To this end, we investigated the effects of gemcitabine in combination with the EPA/DHA mixture, Lipidem™, to establish anti-proliferative and anti-invasive effects in both 2D and 3D culture systems, with specific interest in the incorporation of pancreatic stellate cells to better model the dense desmoplasia which is a characteristic feature of pancreatic cancer.

Materials

Two human pancreatic cancer cell lines, Capan-1 and Panc-1 were obtained from ATCC (Middlesex, UK). An immortalized pancreatic stellate cell line, RLT-PSC was kindly donated by Dr R. Jesenofsky (University of Heidelberg, Germany). All cell lines tested mycoplasma negative on PCR analysis. Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and gemcitabine were obtained from Sigma-Aldrich, (Dorset, UK) and Lipidem™ (DHA/EPA emulsion) from Bbraun (Melsungen, Germany).

Methods

Culture conditions and routine cell culture

Pancreatic cancer cells and pancreatic stellate cells were cultured as adherent monolayers in sterile tissue culture flasks in a humidified atmosphere at 37°C, 5% CO₂. The Capan-1 cells (derived from pancreatic liver metastases) were cultured in Iscove's Modified Dulbecco's Medium (IMDM, GlutaMAX) (Life Technologies Ltd, Paisley, UK), Panc-1 cells (derived from ductal epithelioid pancreatic carcinoma) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, high glucose) (Sigma-Aldrich) and RLT-PSC cells in DMEM/Ham's F-12 (PAA, Somerset, UK). Media was supplemented with 10% Foetal Bovine Serum (FBS), (Life technologies Ltd) except for IMDM which was supplemented with 20 % FBS.

Proliferation assays

Cells were seeded into 24-well plates at a concentration of 5000 cells per well and allowed to adhere for 24 – 48 hours dependant on the cell line. The medium was aspirated and 1 mL of medium containing single reagent or reagent combinations, was added to each well at concentrations ranging from 0 μM to 50 μM for DHA and EPA, and 0 μM to 1 μM for Gemcitabine. Lipidem™ was ready supplied as a suspension and was added at approximate concentrations as per the single agents ie, 10 μM Lipidem™ refers to ~10 μM EPA + 10 μM DHA equivalents. DMSO concentrations were equivocal between all treatments, and did not exceed 0.5%. Cells were then cultured for a further 72, 96, 120 or 144 hours prior to trypsinisation and counting using a Z2 particle counter (Beckman Coulter, Bucks, UK).

Annexin V apoptosis assay

Apoptosis was determined using an Annexin V- FITC Kit (BD Biosciences, NJ, USA). Cells were seeded at a density of 5×10^5 in T75 flasks, allowed to adhere for 24 (Panc-1, RLT-PSC) or 48 hours (Capan-1), and treated for 72 – 144 hours with 10 μM DHA, 10 μM EPA, 10 μM Lipidem™, 2 ηM Gemcitabine, and their combinations. At the end of treatment, medium containing floating cells was reserved and the remaining cells were washed with PBS, trypsinised and pooled with the reserved media. Cells were pelleted (350 x g, 5 min, 4°C) and the pellet resuspended in 10 mL fresh medium supplemented with FBS. Cells were incubated for 30 min at 37°C before being re-centrifuged, and the pellet was resuspended in 1 mL annexin buffer. FITC-conjugated Annexin V (0.05 $\mu\text{g}/\text{test}$) and propidium iodide (0.05 $\mu\text{g}/\text{test}$) were added, followed by a 15 min incubation in the dark at room temperature. All samples were analysed by flow cytometry using a FACS Aria II (Becton Dickinson, New Jersey, USA) with BD FACS Diva analysis software (version 6.1.2).

Cell cycle analysis

Cells were seeded, treated and harvested as per the annexin V apoptosis assay. Pelleted cells were resuspended in 70% ice-cold ethanol and incubated overnight at 4°C. The samples were then pelleted at 350 x g for 10 minutes, the supernatant discarded, the pellet resuspended in 500 μL PBS and ribonuclease A (10 $\mu\text{g}/\text{mL}$) (Sigma-Aldrich) and incubated overnight at 4°C. Propidium iodide was added to a final concentration of 5 $\mu\text{g}/\text{mL}$, and the cells incubated overnight prior to analysis on a BD FACS Aria II with MODFIT LT software.

3D spheroid basement membrane cell invasion assay

The assay was carried out according to the manufacturer's instructions. In brief, cells (3000/well) were resuspended in 1x spheroid formation extracellular matrix (Cultrex, Maryland, USA), and 50 μ L of cell suspension aliquoted in to the Spheroid Formation Plate (Cultrex, Maryland, USA). The plate was centrifuged at 200 x g in a swinging bucket rotor at room temperature, and then incubated at 37°C, 5% CO₂ for 72 hours to promote spheroid formation. The plate was then placed on ice, and 50 μ L of invasion matrix added to each well of the spheroid formation plate. The plate was centrifuged at 300 x g at 4°C for 5 minutes in a swinging bucket rotor to eliminate bubbles and to position the spheroid in the centre of the well, prior to placing at 37°C, 5% CO₂ for one hour to allow the invasion matrix to set. After one hour, 100 μ L of treatment-containing cell culture media was added to the wells and the plate incubated at 37°C, 5% CO₂ for 8 days. Spheroid images were taken using an inverted microscope and pixel area analysed using Image J software.

Organotypic co-culture model of pancreatic cancer

RLT-PSC cells and pancreatic cancer cells were harvested and resuspended at a concentration of 7×10^5 cells /well in 10% FBS-containing cell medium. A gel was made by plating 1 mL of 5.25 volumes of collagen type I (Millipore, Herts, UK): 1.75 volumes of Matrigel™ (Fisher Scientific, Leics, UK): 1 volume of filtered FBS: 1 volume of 10X DMEM and 1 volume of 750,000 PSCs in a 24 well plate. This gel was incubated at 37°C, 5% CO₂ for 1 hour, then 1 mL of 10% DMEM was added on top of the gels and incubated at 37°C, 5% CO₂ overnight.

On day 2, cell suspensions of 125,000 cancer cells/ well or 625, 000 PSCs / well were mixed together. The gels from day 1 were taken out of the incubator and the media carefully removed prior to adding 1 mL of the cell suspension drop-wise on top of the gel. The gels were then incubated overnight at 37°C, 5% CO₂.

Nylon sheets were then pre-coated with 250 μ L of 7 volumes of collagen type I, 1 volume of 10X DMEM, 1 volume of filtered FBS and 1 volume of 10% DMEM. The collagen was allowed to polymerise for 30 minutes at 37°C, cross-linked with glutaraldehyde (Sigma-Aldrich) / PBS and left for 1 hour at 4°C. Membranes were washed in PBS (x3) and medium (x1), covered in medium and left at 4°C overnight.

The following day, the submerged organotypics were lifted onto metal grids covered by the coated nylon sheets in 6 well plates and fed from below. The gels were fed every 24-48 hours with treatment-containing media and harvested on day 10. Gels were then formalin fixed and paraffin embedded.

Immunohistochemistry

Organotypic cultures were assessed by immunohistochemistry for ki67, E-cadherin and β -catenin (Dako, Cambs, UK) which was undertaken using the Novolink Polymer Detection as per manufacturer's instructions. Imaging was undertaken using a Hamamatsu digital slide scanner (Hamamatsu Photonics UK, Herts, UK) with digital magnification.

Cytokine analysis

PDGF-BB was determined in cell culture supernatants using the Quantikine ELISA kit (R&D Systems, Oxfordshire, UK) as per the manufacturers' instructions. One hundred μ L of assay diluent was added to each well of a 96-well sandwich ELISA plate, pre-coated with immobilised PDGFR β /Fc. A standard curve using reconstituted PDGF-BB standard was prepared by serial dilution in the range of 0 – 2000 pg/mL, and 100 μ L of appropriate standard, sample or control added to triplicate wells. Following a 2 hour incubation at room temperature and 4 consecutive washes in wash buffer, 200 μ L of PDGF-BB conjugate was added to each well. The plate was incubated for a further 1.5 hours at room temperature, washed 4 times and 200 μ L of substrate solution added for 30 mins at room temperature in the dark. Fifty μ L of stop solution was added and the optical density measured at 450 nm with wavelength correction at 540 nm using a Fluostar Optima (BMGLabtech, Bucks, UK). Sample concentrations were subsequently determined from the standard curve.

Western blot

Cells were seeded at 1×10^6 cells/9 cm dish and left to adhere overnight (or 48 hours for Capan-1 cells). Cells were stimulated with PDGF-BB (100 ng/mL) (Sigma) for time points up to 5 hours prior to harvesting using Roche complete lysis M buffer supplemented with Phostop phosphatase inhibitor cocktail (Roche, West Sussex, UK). Lysates were analysed by SDS-polyacrylamide gel electrophoresis for PDGFR- β , phospho-PDGFR β , SHP-2, phospho-SHP-2, Akt and phospho-Akt (Cell Signalling Technology, Herts, UK). β -Actin was used as a loading control (Santa Cruz, Heidelberg, Germany).

Results

Effects of Omega-3 fatty acids on cell proliferation, alone and in combination with gemcitabine

Significant growth inhibition was observed following treatments with DHA, EPA, Lipidem™ and gemcitabine (figure 1). Inhibition occurred in a time, dose or cell line dependant manner, with cell lines exhibiting different orders of sensitivity. Following determination of IC₅₀'s at 120 hours via linear regression (table 1), order of sensitivity was determined as follows: For DHA; RLT-PSC>Panc-1>Capan-1; for EPA; Capan-1>RLT-PSC>Panc-1; for Lipidem™; RLT-PSC> Panc-1>Capan-1; for gemcitabine; RLT-PSC>Capan-1>Panc-1.

Treatments were then combined at 10 µM for all omega-3-containing treatments and at 2 nM for gemcitabine (figure 2). In Capan-1 cells, Lipidem™ inhibited growth significantly compared to control and to DHA alone. The combination of Lipidem™ with gemcitabine significantly inhibited growth from 120 hours when compared to DMSO control and gemcitabine alone, and at 144 hours compared to DMSO control, DHA and EPA alone. Combination of Lipidem™ with gemcitabine in the Panc-1 cells resulted in significant growth inhibition compared to DMSO control, DHA and EPA alone from 120 hours. The RLT-PSC cells were most sensitive to the combination treatments. In this cell line, Lipidem™ treatments were significantly different from single agent DHA and EPA treatments across all time points. The combination of the omega-3 fatty acids singly with gemcitabine were all more efficacious than DHA and EPA alone, and more efficacious than gemcitabine alone at 144 hours. The combination of Lipidem™ with gemcitabine significantly decreased cell proliferation compared to single agent treatments of DHA, EPA and gemcitabine.

Effects of Omega-3 fatty acids on apoptosis and cell cycle arrest in combination with gemcitabine

In order to try and ascribe a mechanism by which each drug was eliciting its antiproliferative effects, induction of both apoptosis and cell cycle arrest where investigated.

No significant increase in apoptosis was observed in any of the cell lines at any time point (supporting information S1). Analysis of the combined apoptotic and secondary apoptotic/necrotic cells revealed an increase in overall cell death following treatment with Lipidem™ or Lipdem™ + gemcitabine in the Capan-1, Panc-1 and RLT-PSC cells (figure 3a). No significant growth arrest was observed following any of the treatments (supporting information S2). However, it is likely that anti-proliferative effect with the Lipidem™ + gemcitabine combination was contributed to via GI arrest in Capan-1 cells and RLT-PSC cells (figure 3b).

In addition to anti-proliferative activity, it has been proposed that omega-3 FAs may have anti-invasion activity [12]. In order to assess this, two culture systems were utilised: a spheroid-based 3D mono-culture, and an air-interface organotypic co-culture system which combines pancreatic stellate cells with tumour cells.

Effects of Omega-3 fatty acids on cell migration alone and in combination with gemcitabine
Capan-1, Panc-1 and RLT-PSC cells all showed invasive capacity in the 3D spheroid mono-culture assay (figure 4). All treatments inhibited invasion of Capan-1 cells into the invasion matrix, whereas in Panc-1 and RLT-PSC cells, invasion was inhibited only by the combination of Lipidem™ with gemcitabine. This can be clearly observed by the decrease in branching pseudopodia from each spheroid, but is not reflected in overall spheroid area.

Using the air-interface organotypic co-culture model, it was determined that invasive capacity of Capan-1 but not Panc-1 cells was greatly increased by the presence of pancreatic stellate cells (figure 5a). Addition of Lipidem™ (10 µM) to the Capan-1/RLT-PSC co-culture system resulted in decreased invasion and a decrease in the number and intensity of stained cells for both E-cadherin and β-catenin (figure 5b). Combination of Lipidem™ with gemcitabine did not further enhance this.

In the previous clinical study undertaken using Lipidem™ in combination with gemcitabine [11] treatment-induced changes to cytokine profiles were observed. Here we sought to assess whether this could also be observed within an *in vitro* setting.

Omega-3 fatty acids alone and in combination with gemcitabine, inhibit PDGF secretion from pancreatic cells

Basal levels of PDGF secretion were highest in Capan-1>Panc-1>RLT-PSC (figure 6a). EPA alone significantly reduced PDGF in Capan-1 compared to DMSO control, with the combination of EPA + gemcitabine and Lipidem™ + gemcitabine being the most efficacious. The combination of Lipidem™ + gemcitabine also significantly decreased PDGF levels compared to DHA and EPA alone. In Panc-1 cells, Lipidem™ significantly decreased PDGF levels compared to DMSO control and DHA alone. The combination of Lipidem™ + gemcitabine was again the most efficacious treatment, significantly reducing PDGF levels

compared to both DHA and EPA alone. RLT-PSC cells secreted the lowest levels of PDGF, and were the most refractory to treatment-induced inhibition of PDGF. Treatment with Lipidem™ alone or Lipidem™ + gemcitabine significantly reduced PDGF levels in RLT-PSC cells compared to DHA alone.

PDGF activates the PDGF receptor in pancreatic stellate cells

Despite Capan-1 and Panc-1 cells secreting the highest levels of PDGF, neither cell line expressed the platelet-derived growth factor receptor (PDGFR- β receptor) (figure 6b). RLT-PSC cells expressed PDGFR- β , with maximal stimulation using 100 ng/mL PDGF-BB being observed at 5 minutes. Concurrent stimulation of downstream signalling molecules SHP-2 and Akt was also observed. Conditioned medium from Panc-1 cells, stimulated PDGFR- β phosphorylation in RLT-PSC cells, suggesting functional paracrine signalling between the tumour cells and stellate cells.

Discussion

Previous clinical observations [11] have suggested that the combination of gemcitabine with the combined PUFA emulsion, Lipidem™, enhanced patient quality of life and decreased serum cytokine and growth factor levels, including PDGF, which has a strong association with pro-carcinogenic signalling. Here, we sought to further assess mechanistic consequences of this drug combination *in vitro*, in both pancreatic cancer cell lines, and a pancreatic stellate cell line. It has previously been shown that PUFAs such as DHA and EPA elicit a dose-dependent anti-proliferative effect on a number of pancreatic cancer cell lines, including those that exhibit gemcitabine resistance such as Panc-1 cells (reviewed in [13]), with proliferation further reduced when gemcitabine was combined with single agent PUFAs [14]. Mechanisms of anti-proliferative effect were partially ascribed to reduced NF κ B DNA binding, accompanied by an increase in apoptosis. EPA was also found to induce apoptosis in Panc-1 cells and reduce (cyclooxygenase) COX-2 expression [15], with PUFAs inducing G2/M arrest in MIA PaCa2 cell lines mediated by reduced cdc2 expression [16]. We have similarly shown that DHA, EPA and the combination (Lipidem™) are able to decrease proliferation in a dose and time dependant manner, not only in pancreatic cancer cells, but in a pancreatic stellate cell line. Induction of apoptosis and cell cycle arrest did not reach statistical significance in this study, but notably, previous PUFA studies were all undertaken

at short time points using very high doses of the single omega-3 fatty acids or gemcitabine. These studies are, therefore, more difficult to relate to clinically relevant endpoints.

For combination dosing within the study described here, approximate IC_{25S} for each drug were utilised for up to 144 hours, to ensure that any additive effects would be observed. Interestingly, growth inhibition using the combination of Lipidem™ and gemcitabine was significantly more efficacious in RLT-PSC cells compared to gemcitabine alone, reflected in an increase in the G1 phase of the cell cycle. No studies have previously investigated the effect of PUFAs on pancreatic stellate cells, which play a crucial role in the maintenance and therapeutic resistance of pancreatic cancer.

Recently, the bisphosphonate, zoledronic acid, in combination with nab-paclitaxel was found to induce apoptosis and G1 cell cycle arrest in pancreatic stellate cells *in vitro* and *in vivo* [17], suggesting that a shift in focus towards targeting the pancreatic stellate cell could offer an appropriate therapeutic target. Pancreatic stellate cells are known to contribute to secretion of extracellular matrix (ECM) [18]. They exist in a quiescent state, until they are activated by a variety of cytokines and growth factors such as PDGF [19]. Here, we have shown that both Capan-1 and Panc-1 cell lines produced PDGF, and that tumour cell-conditioned media was able to activate the PDGF receptor and consequent downstream signalling cascades in PSCs, including activation of the pro-proliferative and anti-apoptotic effector kinase, Akt. Furthermore, the combination of Lipidem™ with gemcitabine significantly inhibited PDGF secretion in both of the cancer cell lines. Consequences of PDGF-induced activation of pancreatic stellate cells include increased ECM deposition and desmoplasia, promoting tumour cell growth and invasion via a positive feedback loop. Therefore, interfering with the establishment and continued deposition of this desmoplastic tumour microenvironment by targeting PSCs, may represent an important strategy by which to improve patient outcomes [20]. This is further supported by findings that expression of PDGFRβ (a marker of activated PSCs, but not usually expressed in pancreatic tumour cells), in tumour stroma can act as a prognostic marker in pancreatic adenocarcinoma [21].

Organotypic co-culture models provide a useful tool by which to help further elucidate the role of drug intervention strategies targeting the PSCs. This model, described by Coleman *et al.*, showed that rendering PSCs quiescent, prevented invasion of pancreatic tumour cells into the culture matrix [22]. Here, we established that all treatments were able to inhibit invasion of Capan-1 cells when cultured in mono-culture. Lipidem™-treated media was able to maintain this reduced invasive capacity, even in the presence PSCs. Several

studies have previously investigated a role for the anti-invasive properties of PUFAs. Both DHA and EPA were observed to inhibit PDGF-induced migration of human arterial smooth muscle cells [23], whilst DHA significantly inhibited migration of breast cancer cells *in vitro* and prevented metastasis to the bone *in vivo* [24]. One of the mechanisms by which PUFAs may inhibit invasive processes, is via the downregulation or re-localisation of β -catenin expression. Previous studies have shown that DHA induced degradation of β -catenin in COX-2 overexpressing colorectal tumour cells [25], and downregulated β -catenin in breast cancer xenografts [26]. In keeping with this, we observed that Lipidem™ decreased total and nuclear β -catenin expression in the Capan-1 organotypic cultures, although counterintuitively, E-cadherin was concurrently downregulated. Membrane co-localization of β -catenin/E-cadherin is usually associated with maintenance of an epithelial phenotype, with E-cadherin loss, and β -catenin nuclear translocation associated with an invasive phenotype. However, here, the downregulation of β -catenin, correlated with decreased invasive capacity of the Capan-1 cells. This downregulation may also be a consequence of PUFA-induced abrogation of PDGF secretion. Regulation of β -catenin expression/localisation and the canonical wnt signalling pathway by PDGF is well reported, particularly in fibrotic disease [27-29], and gives further support for use of omega-3 fatty acids in diseases that characteristically feature dense desmoplasia.

There is a growing evidence base advocating the use of PUFAs in combination with standard care chemotherapy for pancreatic cancer. Here we show for the first time, that the EPA/DHA combination (Lipidem™) is able to significantly inhibit proliferation not only in pancreatic cancer cells but also in pancreatic stellate cells. Furthermore, combination of Lipidem™ and gemcitabine completely inhibited PDGF secretion from tumour cells, with tumour-secreted PDGF able to activate PSCs. Invasive capacity of tumour cells when co-cultured with PSCs was abrogated in the presence of Lipidem™, which may be a consequence of downregulated PDGF signalling. Further work to understand precise mechanisms by which Lipidem™ may downregulate pro-tumourigenic PSC signalling, is underway.

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Figure legends

Figure 1. Effect of DHA, EPA, gemcitabine and Lipidem™ on proliferation of a panel of pancreatic cancer cell lines. Black bars represent DMSO control, grey bars represent 10 μM DHA, EPA, Lipidem™ or 0.5 nM gemcitabine. White bars represent 20 μM DHA, EPA, Lipidem™ or 1.0 nM gemcitabine and hatched bars 50 μM DHA, EPA, Lipidem™ or 10 nM gemcitabine. Each experiment was performed in duplicate on 3 separate occasions. Error bars represent SEM. * represents $P \leq 0.05$ compared to DMSO control as determined by oneway ANOVA followed by Tukey's multiple comparison test.

Figure 2. Effect of combination treatments on a panel of pancreatic cancer cell lines. DHA (10 μM), EPA (10 μM), Lipidem™ (10 μM), gemcitabine (2 nM). Each experiment was performed in duplicate on 3 separate occasions. Error bars represent SEM. * represents $P \leq 0.05$ compared to DMSO control, # $P \leq 0.05$ compared to gemcitabine, 'a' $P \leq 0.05$ compared to DHA, 'b' $P \leq 0.05$ compared to EPA as determined by oneway ANOVA followed by Tukey's multiple comparison test.

Figure 3. Effect of combination treatments on a panel of pancreatic cancer cell lines for (A) combined primary and secondary apoptosis and (B) cell cycle, following a 72 hour treatment with DHA (10 μM), EPA (10 μM), Lipidem™ (10 μM), gemcitabine (2nM) and the combinations. Each experiment was performed in duplicate on 3 separate occasions. Error bars represent SEM.

Figure 4. Representative spheroids cultured without invasion matrix (IM), or with invasion matrix in media treated with DHA (10 μM), EPA (10 μM), gemcitabine (GEM) (2 nM), Lipidem™ (LIP) (10 μM) or a combination of gemcitabine+Lipidem™ (L+G). Bar charts show percent change in pixel area for spheroids, analysed by Image J. Each experiment was performed in duplicate on 3 separate occasions. Errors represent SEM. No significant differences were observed in pixel area compared to the DMSO control.

Figure 5. Organotypic co-culture images. (A) Organotypic co-culture model for Capan-1 and Panc-1 cells, alone and in combination with pancreatic stellate cells (RLT-PSC). (B) Representative immunohistochemistry images showing organotypic co-culture of Capan-1

and RLT-PSC treated with Lipidem™. Each experiment was performed on 3 separate occasions. Images created on a Hamamatsu digital scanner using 20x digital zoom.

Figure 6. PDGF analysis in a panel of pancreatic cell lines. (A) Treatment-induced changes to PDGF-BB analysed by ELISA. Treatments were DHA (10 µM), EPA (10 µM), Lipidem™ (10 µM) and gemcitabine (2 nM) alone or in combination. Each experiment was performed in duplicate on 3 separate occasions, errors show SEM. * represents $P \leq 0.05$ compared to DMSO control, 'a' $P \leq 0.05$ compared to DHA, 'b' compared to EPA, as determined by oneway ANOVA followed by Tukey's multiple comparison test. (B) Representative western blots showing effects of PDGF-BB (100 ng/mL) on PDGFR, SHP-2 or Akt phosphorylation over time (minutes). (C) Representative western blot showing RLT-PSC cells treated with Panc-1 conditioned media for 30 minutes or 5 hours. Each experiment was performed on 3 separate occasions.

Supporting information

Figure S1. Induction of apoptosis over time in a panel of pancreatic cancer cell lines. Black bars represent live cells, white bars apoptotic cells and grey bars necrotic cells. Each experiment was performed in duplicate on 3 separate occasions. DHA (10 µM), EPA (10 µM), Lipidem™ (10 µM) gemcitabine (2 nM). Error bars represent SEM. No significant difference was observed.

Figure S2. Induction of cell cycle arrest over time in a panel of pancreatic cancer cell lines. Black bars represent cells in G1, white bars cells in S-phase, and grey bars cells in G2/M. Each experiment was performed in duplicate on 3 separate occasions. Error bars represent SEM. No significant difference was observed.

Table 1. IC₅₀ concentrations for DHA, EPA, Lipidem™ and gemcitabine, generated using 120 hour growth curve data.

	DHA (μM) (±SD)	EPA (μM) (±SD)	Lipidem™ (μM) (±SD)	Gemcitabine (nM) (±SD)
CAPAN-1	47 (±15.6)	16.5 (±6.4)	N/A	4.3 (±4.5)
PANC-1	N/A	N/A	27.7 (±21.5)	6.2 (±0.6)
RLT-PSC	25.7 (±1.2)	23.7 (±2.1)	5.7 (±0.8)	4.1 (±0.5)

N/A = not available due to 50% growth inhibition not being reached at the highest concentration.

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Conflicts of interest:

There are no conflicts of interest to declare. Funding was previously provided to **ARD** by BBraun for a clinical trial using Lipdem™.

Author contributions:

JH: laboratory work, manuscript preparation; **LMH:** study design, managed research, wrote manuscript; **GG:** study design, manuscript preparation, sourced funding; **ARD:** study design, manuscript preparation, sourced funding.