Gemcitabine-fucoxanthin combination in human pancreatic cancer cells

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Abstract. Gemcitabine is a chemotherapeutic agent for pancreatic cancer treatment. It has also been demonstrated to inhibit human pancreatic cancer cell lines, MIA PaCa-2 and PANC-1. The aim of the present study was to investigate the suppressive effect of fucoxanthin, a marine carotenoid, in combination with gemcitabine on pancreatic cancer cells. MTT assays and cell cycle analysis using flow cytometry were performed to study the mechanism of action. The results revealed that combining a low dose of fucoxanthin with gemcitabine enhanced the cell viability of human embryonic kidney cells, 293, while a high dose of fucoxanthin enhanced the inhibitory effect of gemcitabine on the cell viability of this cell line. In addition, the enhanced effect of fucoxanthin on the inhibitory effect of gemcitabine on PANC-1 cells was significant ($P<0.01$). Fucoxanthin combined with gemcitabine also exerted significant enhancement of the anti-proliferation effect in MIA PaCa-2 cells in a concentration dependent manner ($P<0.05$), compared with gemcitabine treatment alone. In conclusion, fucoxanthin improved the cytotoxicity of gemcitabine on human pancreatic cancer cells at concentrations that were not cytotoxic to non-cancer cells. Thus, fucoxanthin has the potential to be used as an adjunct in pancreatic cancer treatment.

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Introduction

Pancreatic cancer is the 8th most common cause of cancer-related deaths worldwide due to its poor prognosis and difficulty of diagnosis and treatment (1). Mortality rates have remained constant over the past three decades. Chemotherapy is the mainstay treatment for the 15-20% of patients whose pancreatic tumours can be effectively removed surgically, as well as for patients with tumours that are not surgically resectable (2). However, >90% of pancreatic cancer cases are resistant to current chemotherapies (3,4). At present, the DNA synthesis inhibitor, gemcitabine, (2',2'-difluoro-2'-deoxycytidine) is used as the preferred chemotherapeutic agent (5,6), despite the fact that even when combined with other chemo- or radio-therapeutic agents, it exhibits limited efficacy, and produces severe adverse reactions (5,7). Gemcitabine can be toxic to healthy cells, resulting in unpredictable severe toxic effects. The inherent biological characteristics of pancreatic cancer, as well as the blood-pancreas barrier, possibly contribute to the unsatisfactory therapeutic effects of gemcitabine (8).

Thus, there is a need for more effective anticancer drugs which have lesser toxic side effects to improve clinical efficiency.

In the present study, fucoxanthin, a natural product first isolated from the marine seaweeds *Fucus*, *Dictyota*, and *Laminaria* in 1914, and thus one of the most abundant carotenoids, was examined (9). Fucoxanthin is found in both macroalgae such as *Undaria pinnatifida* or *Laminaria japonica*, as well as microalgae such as *Phaeodactylum tricornutum* or *Cylindrotheca closterium* (10). Fucoxanthinol, the main fucoxanthin metabolite, has been detected in human plasma after the daily intake of wakame (Japanese common name for *U. pinnatifida*). Its bioavailability and metabolism are higher in humans than in mice (11) and does not exhibit any significant adverse effects *in vivo* (12). It has been regarded as a potential natural substance for cancer treatment (9). Although human experiments on the effects of fucoxanthin are lacking, animal experiments have shown evidence of anticancer effects. Oral administration of fucoxanthin has revealed no toxicity and mutagenicity (13-17). Apart from anticancer effects, fucoxanthin has also been demonstrated to have other health beneficial effects (18-22). Fucoxanthin uses various mechanisms to suppress tumour formation; inducing autophagy (21,22), arresting the cell cycle at the G1/G0 phase, inducing apoptosis, enhancing gap junctional intercellular communication, and involving different regulatory events in SAPK/JNK, Akt/mTOR, Bcl-2, JAK/STAT, MAPK and NF-κB pathways (23,24). Fucoxanthin has been shown to enhance chemotherapeutic efficacy of cisplatin *in vitro* (25). Recently, the combination therapy (chemotherapy in combination with fucoxanthin) has been evaluated in colon and liver cancers (25-29), lung cancer (30), breast cancer (31), and cervical cancer (32).

Pancreatic ductal adenocarcinoma accounts for >90% of all pancreatic cancers (33), and MIA PaCa-2 and PANC-1 cell lines, derived from primary tumours, are currently used as *in vitro* models to study pancreatic ductal adenocarcinoma carcinogenesis (34,35).

The aim of the present study was to investigate the suppressive effect of fucoxanthin on the growth of pancreatic cancer cell lines, MIA PaCa-2 and PANC-1, and the effect of combining it with gemcitabine. The 293 cell line, as a reference line for potential toxicity towards a non-cancer cell line, and MIA PaCa-2 and PANC-1 cell lines were utilised in the present study to determine the various cytotoxic effects of treatment between pancreatic cancer cells and a control. Flow cytometry was used to determine the possible mechanism of action by analysing the cell cycle and apoptosis.

Materials and methods

Materials. Fucoxanthin (cat. no. F6932) and MTT (cat. no. M2128) were purchased from Sigma-Aldrich NZ; Merck KgaA. Human pancreatic cancer cell lines, MIA PaCa-2 (cat. no. CRL-1420TM) and PANC-1 (cat. no. CRL-1469TM), and human cell line, 293 (cat. no. CRL-1573TM) were purchased from American Type Culture Collection (ATCC). Cell culture medium (RPMI-1640), penicillin-streptomycin and L-glutamine were purchased from Thermo Fisher Scientific, Inc. Fetal bovine serum was purchased from Moregate BioTech. All three cell lines were stored in liquid nitrogen. Following thawing, the cell lines were maintained in a tissue culture flask containing complete growth culture medium (RPMI-1640) with 1% of L-glutamine, 1% of penicillin-streptomycin and 10% of fetal bovine serum. All cells were cultured in an incubator at 37˚C, with 5% carbon dioxide humidified air.

MTT assay. MTT assays were used to determine cell viability (36). Cells were seeded at densities of 5,000 cells/well in 96-well plates, for 6-24 h and then cultured in the incubator at 37˚C for the following experiment. A total of 100 µl of fresh complete culture medium containing various concentrations of drugs (i.e., gemcitabine 25, 50 and 500 nM) was added to corresponding wells. Following incubation for 0, 24, 48 and 72 h, the medium was carefully removed and replaced with 100 µl of fresh complete culture medium. An aliquot of 10 µl of MTT stock solution (0.2 mg/ml) was added to each well and the plates were placed in the 37˚C incubator for 2 h. The supernatant was gently removed from the wells. An aliquot of 150 µl of DMSO was added to each well and mixed thoroughly using an orbit plate shaker. After incubating at 37˚C for 20-30 min, the plate was shaken briefly and the absorbance was measured by a plate reader (FLUOstar Omega; Alphatech Systems, Ltd.), at 540 nm. Background subtraction was utilised for generation of final values.

Determination of the effect of fucoxanthin. The cell density selected in this study for MIA PaCa-2, PANC-1 and 293 cells was 5,000 cells/well, according to our preliminary culture study. Following proper dilution, a multi-channel pipette was used to seed 100 µl of cells/well into the 96-well plate. The fucoxanthin stock solution was prepared by dissolving fucoxanthin in absolute ethanol with its concentration as 5 mM. Fucoxanthin (prepared in absolute ethanol) was diluted with cell culture medium. The range of concentrations of fucoxanthin were different for the three cell lines: MIA PaCa-2 (fucoxanthin concentration from 0.02 to 1 µM); PANC-1 (from 0.5 to 100 µM) and 293 (from 1 to 100 µM). Since fucoxanthin was dissolved in ethanol, the effect of ethanol alone on the cells was determined. The results revealed that 2, 1.6 and 1.0% fucoxanthin inhibited the growth of MIA PaCa-2 cells after incubating for 24, 48 and 72 h. Ethanol at a concentration of ≤0.5% did not significantly affect the growth of MIA PaCa-2 cells. No effect on the growth of PANC-1 cells cultured with ethanol controls was noted. The study was then carried out using fucoxanthin concentrations under 25 µM, and 100 µl of each diluted fucoxanthin solution was added to wells immediately for analysis.

Determination of the colour effect of fucoxanthin on the absorbance value (OD value). As fucoxanthin is an orange-coloured pigment, its colour may impact the final absorbance value of each well when performing MTT assays. Different concentrations of fucoxanthin solution were prepared to assess respective absorbance values. The highest fucoxanthin concentration used was 100 µM and two test methods were used for this study. The first was performed by testing 100 µl of each fucoxanthin solution under the plate reader directly; and the other method was carried out by following the MTT assay protocol. A total of 10 µl MTT solution was added to each well and incubated at 37˚C for 4 h, then 150 µl DMSO was added and finally the absorbance was read at 540 nm. According to

the results, fucoxanthin solution was replaced with fresh cell culture medium before the addition of the MTT solution in the present study.

Determination of optimum concentration of gemcitabine. According to previous single treatment experiment results, the optimum concentrations of fucoxanthin and gemcitabine were determined. In the combination treatment experiment, fucoxanthin at concentrations of 150, 250 and 300 nM, and gemcitabine, at 25 and 50 nM were concurrently combined with each other to treat MIA PaCa-2 cells. In addition, fucoxanthin concentrations (10 and 20 μM) were combined with gemcitabine (50 and 500 nM, respectively) to treat PANC-1 cells. An MTT assay was applied to determine the cell viability, and the experimental steps were the same as those aforementioned.

Synergism analysis. The nature of the combination between gemcitabine and fucoxanthin was quantified by synergism quotient (SQ) (37-39). SQ is defined as the net growth inhibitory effect of the combination treatment by the sum of the net individual treatment effect on growth inhibition. A quotient of >1.1 indicates a synergistic effect, a quotient between 0.9 and 1.1 indicates an additive effect, while a quotient of <0.9 indicates an antagonistic effect (38).

Cell cycle assay. After detaching, counting and diluting, cells were seeded onto 6-well plates. The seeding density for all cell lines in this study was 50,000 cells/ml and a 2-ml cell solution was seeded in each well. The plates were maintained in a 37˚C incubator for 6-24 h to ensure that almost all of the cells were detached from the walls of the wells.

When the cell attachment rate was high, cells were treated with 2 ml of serum-free medium (no FBS, with 1% penicillin and 1% L-glutamine) in each well, for synchronizing cell proliferation. One plate set in this study was a control group and the other plates were designed as treatment groups. In Plate 1 (control group), wells A, B and C were treated with 2 ml of serum-free medium (no FBS), and wells D, E and F were filled with 2 ml of complete culture (10%) medium. Before adding the fresh medium (serum-free medium or 10% FBS medium), the old medium was removed first. The fresh medium was added slowly and gently in circles along the wall of the well. For Plate 2 (treatment group), all the wells were treated with 2 ml of serum-free medium. The plates were maintained in the 37˚C incubator for 24 h.

All treatments were maintained in 15 ml centrifuge tubes which were prepared with complete culture (10% FBS) medium. The old serum-free medium was first removed gently, and then 2 ml of well-mixed treatment solution was carefully added into the designated wells. A total of 2 ml complete culture medium was added to the control well. All plates were maintained in the incubator for 48 h (day 2) and 72 h (day 3). Control cells were collected from Plate 1 with day 0 samples. Namely, fucoxanthin, 150, 250 and 300 nM, and gemcitabine, 25 and 50 nM, were used to treat MIA PaCa-2 solely and jointly. For PANC-1 cells, fucoxanthin concentrations were 10 and 20 μM and gemcitabine were 50 and 500 nM.

Cell cycle analysis. The permeabilizing solution of (total volume) 1 ml per test tube consisted of: 0.1% Triton X-100 (1 μl for each 1 ml) + RNAse A (50 μg/ml from stock solution of 1 mg/ml). For example, 20 tubes were formulated and dispensed as follows: 20 μl Triton X-100 + 1,000 μl RNAse +19,980 μl PBS. After centrifuging all tubes at 125 x g for 2 min at 0-4˚C, the ethanol was gently removed, and 3 ml ice cold PBS was added to each tube before the second centrifugation step. Following the second centrifugation (125 x g for 2 min at 0-4˚C), another 3 ml of ice-cold PBS was added to each tube to replace the initial wash. In total, the cells were washed twice with 3 ml ice cold PBS. In the process of PI staining, the supernatant in each well was gently removed and then 1 ml of well-mixed permeabilizing solution was added to each tube. All cells in the tubes were carefully mixed before being transferred to test tubes and then incubated at 37˚C for 30-45 min. Following permeabilization, 5 μg/ml of PI (5 μl to 1 ml in each tube) was added to each test tube and incubated for 5 min. Finally, all the tubes were analysed under a flow cytometer (MoFlo XDP Beckman Coulter, Inc.).

The IC50 values were calculated using PRISM® software (version 6.0; GraphPad Software, Inc.), and the IC50 values were obtained using dose-response inhibition, nonlinear regression (curve fit): Log (inhibitor) vs. response-variable slope (four parameters). Kaluza® Flow Cytometry Analysis Software (version 1.3; Beckman Coulter, Inc.) was applied in cell cycle results analysis to measure the cell cycle distributions in the present study.

Statistical analysis. All experiments in the present study were performed at least three times. Data were assessed for normal distribution before analysis. Statistical differences in multiple groups were determined by one-way analysis of variance (ANOVA) with PRISM® software (version 6.0; GraphPad Software, Inc.) and SPSS (version 22.0; IBM Corp.). Statistical comparisons were performed using Tukey's post hoc test. Analysis between two groups was determined using unpaired Student's t-test. Data are expressed as the means ± standard deviation (SD; sample n=3 with triplicate analysis performed on each sample). P<0.05 was considered to indicate statistically significant differences.

Results

In order to analyse the treatment effect of gemcitabine or fucoxanthin on pancreatic cancer cell lines MIA PaCa-2, PANC-1, and 293 cell growth, cells were treated *in vitro* in a dose or time-and-dose course experiment. Experiments were performed in triplicate and repeated at least three times independently (Table I).

Inhibitory effect of gemcitabine or fucoxanthin on MIA PaCa-2 pancreatic cancer cells. Culture of cells with various concentrations of gemcitabine for 72 h resulted in significant suppression of cell viability in a dose-dependent manner. ANOVA analysis revealed that cell viability was statistically and significantly decreased with the increasing concentration of gemcitabine, thus the concentration of gemcitabine was a significant factor in altering cell viability (P<0.01); the IC_{50} was 25.00±0.47 nM after 72 h of treatment (Table I). Gemcitabine at 25 and 50 nM exhibited approximately 63.45 and 42.18% cell viability of MIA PaCa-2 cells at 48 h, respectively (Table II).

Table I. Cytotoxicity (IC_{50}) of treatment in MIA PaCa-2 cells, PANC-1 and 293 cells detected at various time points: gemcitabine (72 h) or fucoxanthin (24, 48 and, 72 h).

	IC_{50} (μM) aftergem-citabine treatment	IC_{50} (μM) after fucoxanthin treatment		
Cell line	72 h	24 h	48 h	72 h
MIA PaCa-2	25.00±0.47	17.72±1.04	10.68±0.64	8.74±0.28
PANC-1	48.55±2.30	N/A	N/A	10.58±0.56
293 Cells	48.82±3.27	28.97±1.58	8.70±1.17	8.28±0.30

Data are presented as the mean ± SD (n=6). IC_{50}, half maximal inhibitory concentration.

Table II. Cell viability percentages of MIA PaCa-2 cells after 72 h of incubation with gemcitabine, fucoxanthin, or gemcitabine-fucoxanthin combination.

Compound Fucoxanthin (nM)	Gemcitabine (nM) 0 (%)	25 (%)	50 (%)
0	100	63.45±0.54	42.18±0.48
150	99.8±0.19	59.76±1.63[a]	39.81±0.69[a]
250	98.99±0.89	53.06±0.70[a]	38.32±0.52[a]
300	99.83±0.61	50.40±0.50[a]	36.72±0.12[a]

Data are presented as the mean ± SD (n=6). [a]P<0.01 (Student's t-test).

These two concentrations were selected in the following combination study.

Fucoxanthin inhibited the proliferation of MIA PaCa-2 cells in a concentration-dependent manner (Table II) at 72 h. One-way ANOVA revealed that cell viability was statistically and significantly decreased with the increasing concentration of fucoxanthin treatment (P<0.01) after treatment for 72 h. The IC_{50} values within the three days of treatment significantly and statistically decreased to 17.72±1.04 (24 h), 10.68±0.64 (48 h) and 8.74±0.28 μM (72 h) (Table I). Morphological changes observed microscopically became more marked with increasing culture time, in the presence of fucoxanthin concentrations >6.25 μM. Cells diminished in size and were scattered and more easily detached. After 72 h, the volume of cells was significantly reduced, and the edges of the cells were rough (data not shown).

Inhibitory effect of gemcitabine or fucoxanthin on PANC-1 pancreatic cancer cells. Gemcitabine was effective in inhibiting PANC-1 cells at only 72 h of incubation in a dose-dependent manner, yielding an IC_{50} value of 48.55±2.30 nM (Table I). ANOVA revealed that cell viability was statistically and significantly decreased with the increasing concentration of gemcitabine treatment, thus, the alteration of treatment concentration was associated with a change in cell viability.

Furthermore, fucoxanthin inhibited proliferation of PANC-1 cells in a dose-dependent manner after 72 h incubation. Only 72-h incubations were performed in view of the aforementioned result of gemcitabine. The IC_{50} value was 10.58±0.56 μM (Table I). ANOVA revealed that treatment with fucoxanthin was a significant factor in the inhibition of cell growth with increasing treatment concentrations and exposure times.

Inhibitory effect of gemcitabine and fucoxanthin on 293 cell line. The same gemcitabine concentrations used for MIA PaCa-2 cells were used for 293 cells. Gemcitabine significantly inhibited cell viability in a concentration-dependent manner. The IC_{50} value was 48.82±3.27 nM (72 h) (Table I). Fucoxanthin inhibited 293 cell growth at concentrations in a dose- and time-dependent manner. The IC_{50} values showed a significant decrease after three days of treatment to 28.97±1.58 (24 h), 8.70±1.17 (48 h), and 8.28±0.30 μM (72 h), respectively (Table I). ANOVA indicated that the treatment with gemcitabine or fucoxanthin was a significant factor in the inhibition of cell growth with increasing treatment concentrations and exposure times.

Inhibitory effect of gemcitabine-fucoxanthin combination. Based on single drug experiments, 72 h of incubation time for combination drug treatment was used for MIA PaCa-2 cells. The cell viability in groups treated with combination of gemcitabine and fucoxanthin for 72 h, is presented in Table II. The cell viability was not markedly altered with fucoxanthin treatment alone (concentrations from 0 nM to 300 nM). However, gemcitabine 25 nM combined with 150, 250 and 300 nM fucoxanthin significantly reduced the cell viability by approximately 4, 10 and 13%, respectively, as compared with gemcitabine alone (cell viability, 63.45±0.54%). Similarly, in the gemcitabine 50-nM treatment group, the enhanced inhibitory effect of the combination treatment with fucoxanthin was also observed: Gemcitabine treatment 50 nM alone (42.18±0.48%) vs. gemcitabine treatment with increasing concentrations of fucoxanthin at 150 (39.81±0.69%), 250 (38.32±0.52%) and 300 nM (36.72±0.12%). According to the statistical analysis, single treatment with fucoxanthin alone could not significantly decrease the cell viability (P>0.01) at treatment concentrations

Table III. Synergism analysis of MIA PaCa-2 cells after 72 h of incubation with combination treatment of gemcitabine-fucoxanthin.

Compound Fucoxanthin (nM)	Gemcitabine (nM)		
	0	25	50
0	N/A	N/A	N/A
150	N/A	1.079	1.034
250	N/A	1.205	1.033
300	N/A	1.316	1.085

Data are presented as the mean ± SD (n=6).

Table IV. Cell viability percentages of PANC-1 cells after 72 h of incubation with gemcitabine, fucoxanthin, or gemcitabine-fucoxanthin combination.

Compound Fucoxanthin (nM)	Gemcitabine (nM)		
	0 (%)	50 (%)	500 (%)
0	100	90.20±0.67	82.15±0.88
10	56.91±3.00[a]	52.92±0.63[a]	49.42±2.51[a]
20	18.54±1.10[b]	17.42±1.20[b]	16.01±0.36[b]

Data are presented as the mean ± SD (n=6). [a]P<0.01 and [b]P<0.001 (Student's t-test).

Table V. Synergism analysis of PANC-1 cells after 72 h of incubation with combination treatment of gemcitabine-fucoxanthin.

Compound Fucoxanthin (nM)	Gemcitabine (nM)		
	0	50	500
0	N/A	N/A	N/A
10	N/A	0.889	0.823
20	N/A	0.902	0.840

Data are presented as the mean ± SD (n=6).

of 150, 250 and 300 nM. However, gemcitabine treatment alone at 25 and 50 nM significantly decreased the cell viability (P<0.01). Combination treatment also significantly decreased the cell viability (P<0.01). Additionally, in the presence of fucoxanthin, the cell viability was significantly decreased in a dose-dependent manner as compared to gemcitabine treatment alone (P<0.01). As revealed in Table III, treatment with gemcitabine at 25 nM with increasing concentrations of fucoxanthin from 150 to 300 nM, yielded an SQ value which increased from 1.079 (additivity) to 1.316 (synergism). Gemcitabine, at 50 nM, with increasing concentrations of fucoxanthin, increased the SQ value from 1.034 (additivity) to 1.085 (additivity). These results indicated that treatment with fucoxanthin generated a synergistic effect at a combined treatment with 25 nM of gemcitabine. In all groups, with increasing concentrations of fucoxanthin, the cell viability of MIA PaCa-2 cells decreased. Hence, fucoxanthin was able to enhance the inhibitory effect of gemcitabine on cell viability of MIA PaCa-2 cells in a concentration-dependent manner, even at a low concentration range. The single treatment results revealed that PANC-1 cells were not sensitive to gemcitabine as compared to MIA PaCa-2 cells. Therefore, gemcitabine at 50 and 500 nM was used in combination with fucoxanthin at 10 and 20 μM. The treatment time was also 72 h.

The PANC-1 cell viability values under different concentrations of drugs are listed in Table IV. After 72 h of incubation, with 10 and 20 μM fucoxanthin alone, the number of viable cells decreased to 56.91±3.00 and 18.54±1.10%, respectively. Treatment with gemcitabine alone decreased the number of viable cells. However, even at a concentration of gemcitabine increased 10 times from 50 to 500 nM, the cell viability was minimally affected (from ~10 to 18%, respectively). This effect was observed in all combination treatments. In the combination treatment group (fucoxanthin, 10 μM), the addition of gemcitabine at concentrations of 50 and 500 nM did not significantly reduce (~3%) the viability of cancer cells. Once increased, a fucoxanthin concentration of 20 μM, demonstrated only a 2% increase in the inhibition rate at gemcitabine concentrations of 50 and 500 nM. The single treatment of either fucoxanthin alone or gemcitabine alone decreased the cell viability. However, the combination treatment did not generate a significant effect for reduced cell viability (P>0.01). As noted in Table V, the concentration of fucoxanthin (from 10 to 20 μM) increased the SQ values in this stated range: 50 nM gemcitabine [0.889 (antagonistic effect) to 0.902 (additive effect), respectively] and 500 nM [0.823 (antagonistic effect) to 0.840 (antagonist effect)].

Gemcitabine significantly inhibited the cell viability of 293 cells by approximately 23, 49 and 76% after 72 h of incubation at 25, 50 and 500 nM concentrations, respectively (Table VI; P<0.05). In the fucoxanthin alone groups (150, 250, 300, 10,000 and 20,000 nM), there was no significant suppression by fucoxanthin at ≤300 nM, but there was significant inhibition at high concentrations of 10 and 20 μM, as compared to the control (P<0.01). In the combination treatment groups, adding fucoxanthin at concentrations of 0 to 150 nM, slightly increased cell viability (2.58 and 4.05%; P<0.05) at gemcitabine treatment concentrations of 25 and 50 nM, respectively. No significant differences in cell viability were observed at gemcitabine treatment concentrations of 25 and 50 nM combined with fucoxanthin treatment concentrations of 150 to 300 nM (P<0.01). However, combination with high concentrations of fucoxanthin (10 and 20 μM), induced significantly greater inhibition than the gemcitabine alone group (P<0.01). This may be due to the cytotoxicity of fucoxanthin used alone at high concentrations (Table VI). In Table VII, gemcitabine (25 nM) had an SQ value increase from 0.833 (antagonistic effect) to 1.087 (additive effect) as the concentration of fucoxanthin increased from 150 to 300 nM. Consistently, the SQ value increased from 0.900 (additive effect) to 0.918 (additive effect) with increasing fucoxanthin concentrations of 150 to 300 nM in the gemcitabine 50-nM groups. However,

Table VI. Cell viability percentages of 293 cells after 72 h of incubation with gemcitabine, fucoxanthin, or gemcitabine-fucoxanthin combination.

Compound Fucoxanthin (nM)	0 (%)	Gemcitabine (nM) 25 (%)	50 (%)	500 (%)
0	100	77.42±2.34[a]	51.27±0.88[b]	23.60±1.79[c]
150	99.08±1.71	80.00±5.80	55.32±2.20	
250	99.06±1.23	79.02±4.49	55.80±2.49	
300	100.04±1.99	75.92±4.77	55.73±2.31	
10000	30.51±2.92[b]		22.47±2.30[b]	20.04±1.45
20000	12.72±0.66[c]		11.25±1.03[c]	9.89±0.93[b]

Data are presented as the mean ± SD (n=6). [a]P<0.05, [b]P<0.01 and [c]P<0.001 (Student's t-test).

Table VII. Synergism analysis of 293 cells after 72 h of incubation with combination treatment of gemcitabine-fucoxanthin.

Compound Fucoxanthin (nM)	Gemcitabine (nM) 0	25	50	500
0	N/A	N/A	N/A	N/A
150	N/A	0.833	0.900	
250	N/A	0.875	0.900	
300	N/A	1.087	0.918	
10000	N/A		0.655	0.559
20000	N/A		0.649	0.565

Data are presented as the mean ± SD (n=6).

once the concentration of fucoxanthin increased to 10 mM, the SQ value decreased below 0.700 (antagonistic effect).

Combination of gemcitabine and fucoxanthin on the cell cycle. The cell cycle distribution of MIA PaCa-2 cells is presented in Fig. S1. According to Fig. S1 the G0-G1 phase was significantly increased from 41.04% (control) to 60.37% (24-h serum-starved cells) and the G2-M and S phases were decreased from 27.00% (control) to 15.75% (24-h serum-starved cells) and 17.28% (control) to 10.62% (24-h serum-starved cells), respectively. The distribution of the cell cycle was not altered by incubation with 150 or 250 nM fucoxanthin alone for either 48 or 72 h (Table SI). However, fucoxanthin at a concentration of 300 nM has a slight effect: The percentage of the G_0-G_1 phase increased ~1.9 and 3.75% at both 48 and 72 h, respectively. Treatment with 10 μM fucoxanthin for 48 h, resulted in an increase of MIA PaCa-2 cells in G_0-G_1 phase (from 48.78 to 59.51%), and the percentage of sub-G_1 was increased (from 8.0 to 12.83%). At 72 h, fucoxanthin (10 μM) not only increased the cells in sub-G_1 phase, but also increased the percentage of cells in the G_2-M phase with a concomitant decrease in the number of cells in the S phase (Table SI). Gemcitabine (at 25 and 50 nM) caused a significant accumulation of cells in the S phase, as compared to the control at both 48 and 72 h, while the number of cells in the G_0-G_1 phase was reduced (Table SI). Additionally, sub-G_1 phase accumulation increased in a time- and dose-dependent manner, particularly with 50-nM gemcitabine treatment at 72 h.

In the combination treatment groups, the results indicated that fucoxanthin (150, 250 and 300 nM) combined with gemcitabine in MIA PaCa-2 cells, for 48 h, increased the percentage of cells in the S phase, but the cells in the sub-G_1 phase were not altered (Table SI), as compared to gemcitabine (25 and 50 nM) alone (Fig. 1A). At the 72-h combination treatment, the sub-G_1 percentage was enhanced with the corresponding increase in fucoxanthin concentration. However, sub-G_1 phase accumulation in the 25-nM gemcitabine combination groups was more apparent than the 50-nM gemcitabine groups (Fig. 1B).

Effects of gemcitabine and fucoxanthin on PANC-1 cell cycle progression. The cell cycle distribution of PANC-1 cells is presented Table SII. In addition, cell starvation (medium-only, without serum) for 24 h, synchronised and blocked the PANC-1 cells in G_0-G_1 phase (Fig. S2). The percentage of cells in the G_0-G_1 phase was increased by ~19%, while the number of cells in the S phase decreased from 20.73 to 9.62% and the number of cells in the G_2-M decreased from 24.34 to 18.03%. In the groups treated only with fucoxanthin (10 or 20 μM) for 48 h, the results revealed that the accumulation of cells in the G_0-G_1 phase was significantly increased compared to the control (Table SII). Furthermore, the percentage of cells in the S phase decreased with the increase of fucoxanthin concentration. At 72 h, fucoxanthin 20 μM did not block the cells in the G_0-G_1 phase. However, fucoxanthin induced the increase of the percentage of cells in the sub-G_1 phase (24.70%) compared with the control cells (9.00%) at 72 h (Table SII). Fucoxanthin decreased the proportion of cells in the G_2-M phase and increased the percentage of sub-G_1 cells in a time- and dose-dependent manner. Gemcitabine, 50 nM, 500 nM and 50 μM blocked the cells in the G_0-G_1 phase at 48 h, and the percentage of cells in the sub-G_1 phase was increased in a time- and dose-dependent manner. Similar results were obtained at 72 h, except for gemcitabine at a concentration of 50 μM which induced cell apoptosis [56.65 (control) vs. 25.99% (50 μM gemcitabine); Table SII)].

As revealed in Table SII, in the combination treatment groups (50 and 500 nM gemcitabine combined with 10 μM

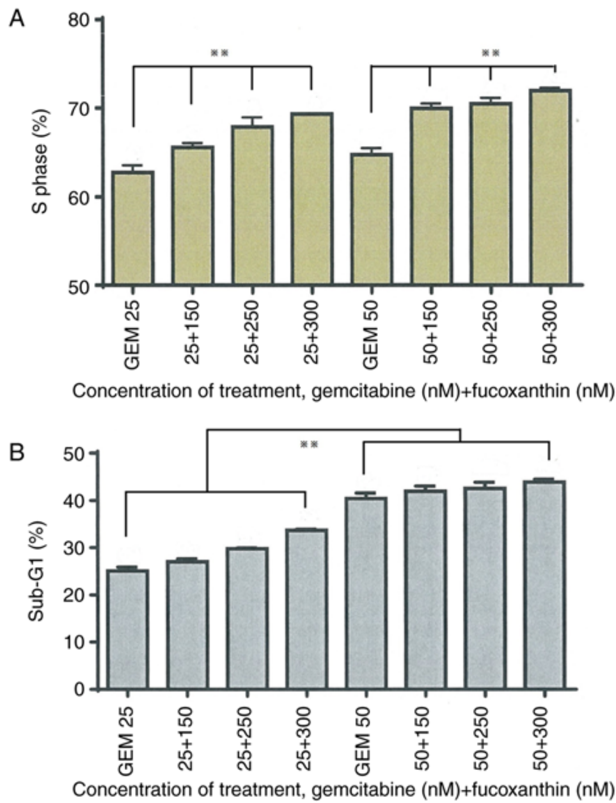

Figure 1. Effects of fucoxanthin on gemcitabine-induced cell cycle arrest in MIA PaCa-2 cells. (A) Percentage of MIA PaCa-2 cells in the S phase after treatment for 48 h. (B) Percentage of MIA PaCa-2 cells in the sub-G1 phase after treatment for 72 h. Data are presented as the mean ± SD (n=3). **P<0.01 (ANOVA with post hoc Tukey's test).

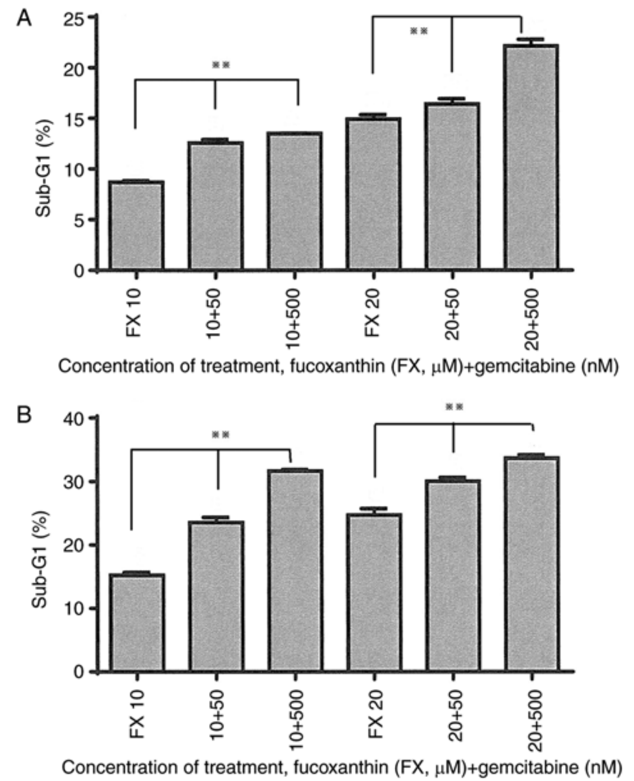

Figure 2. Effects of fucoxanthin on gemcitabine-induced cell cycle arrest in PANC-1 cells. (A) Percentage of PANC-1 cells in the sub-G1 phase after treatment for 48 h. (B) Percentage of PANC-1 cells in the sub-G1 phase after treatment for 72 h. Data are presented as the mean ± SD (n=3). **P<0.01 (ANOVA with post hoc Tukey's test).

fucoxanthin), an increase in the percentage of cells in the G_0-G_1 phase as well as in the sub-G_1 phase was observed, compared with the cells treated only with 10 μM fucoxanthin for 48 h. The accumulation of cells in the S phase increased with higher gemcitabine concentrations (50 to 500 nM), and changes in the G_2-M phase were negligible. However, cells treated with the combination drugs for 72 h did not exhibit increased arrest of the G_0-G_1 phase, with respect to cells treated only with fucoxanthin 10 μM. By contrast, the percentage of cells in the sub-G_1 phase increased after treatment for 48 and 72 h (Fig. 2A and B). As for cells treated with 20 μM fucoxanthin combined with gemcitabine (50 and 500 nM), the combined treatment induced increased accumulation of cells in the S phase and cell apoptosis, as compared to cells treated only with 20 μM fucoxanthin, at both at 48 and 72 h (Table SII). G_2-M phase detection was negligible in the fucoxanthin 20-μM combination treatment groups at both time points, similar to the 10-μM fucoxanthin combination treatment groups (Table SII).

Effects of gemcitabine and fucoxanthin on 293 cell cycle progression. The results of cell cycle distribution revealed that 24-h starvation (medium-only, without serum) did not arrest 293 cells in the G_0-G_1 phase; it was demonstrated that the percentage of serum-starved cells in the G_0-G_1 phase was slightly decreased compared with the control (Fig. S3). The cell cycle distribution of 293 cells is presented in Table SIII. In the single treatment groups, the distribution of cell cycle phases in fucoxanthin-treated (150, 250 and 300 nM) 293 cells was similar to that in control cells at both 48 and 72 h after treatment. Cells treated only with fucoxanthin at a concentration of 10 μM at 48 h induced a 1.53% higher cell apoptosis value than the control cells, and the percentage of cells in the G_0-G_1 phase was even lower than that of control cells. The G_2-M and S phases were not markedly altered in relation to the controls. However, at 72 h, 10 μM fucoxanthin was responsible for a higher accumulation of cells in the G_2-M phase, and its percentage of sub-G1 cells was only ~2% higher than the same concentration at 48 h. There were no significant differences in the cell cycle distribution of 25-nM gemcitabine-treated cells, at 48 h, in relation to the control, and apoptosis in sub-G1 was increased by only ~6%. However, treatment with 50-nM gemcitabine induced apoptosis from sub-G1 (25.28%), and most of the cells were arrested at the S phase. At 72 h, gemcitabine at a concentration of 25 nM increased the percentage of sub-G_1 over a small range as compared with control group. Gemcitabine, at a concentration of 50 nM, induced cell apoptosis (sub-G1) of up to 38.57% and no cell phase distribution could be observed, implying a high level of apoptotic induction in these cells (Table SIII).

In the combination treatment groups [fucoxanthin (150, 250 and 300 nM) combined with gemcitabine (25 and 50 nM), for 48 and 72 h], no significant differences in the distribution of all phases in the cell cycle were revealed, as compared to cells treated only with gemcitabine. Combination treatments induced a slightly lower percentage of cell apoptosis compared to that observed with single-gemcitabine treatment.

Discussion

To the best of our knowledge, this is the first study, investigating the effects of fucoxanthin and its combination with gemcitabine in pancreatic cancer cells. The characteristics of high anticancer efficacy, with mild or no toxicity to normal cells, is the primary objective in cancer research. Fucoxanthin used alone in treatment has exhibited significant anticancer efficacy (31), however, when used in combination with gemcitabine as determined in the present study, was more effective with less harmful effects to normal cells.

The results obtained in the present study indicate that the inhibitory effect of gemcitabine on MIA PaCa-2, PANC-1 and 293 cells occurs in a dose-dependent manner. This is consistent with previous studies of gemcitabine on, pancreatic ductal adenocarcinoma cell lines MIA PaCa-2 and PANC-1 (39,40), breast adenocarcinoma cell lines MCF-7 and MDA-MB-231 (41), colorectal adenocarcinoma cell line HT-29 (42), and cervical carcinoma cell lines (43). The IC_{50} values for gemcitabine determined in this study were 25.00±0.47 nM for MIA PaCa-2 cells, 48.55±2.30 μM for PANC-1 cells and 48.82±3.27 nM for 293 cells. These three different IC_{50} values indicated a different sensitivity of the three cell lines to gemcitabine. MIA PaCa-2 cell line was the most sensitive to treatment of gemcitabine. PANC-1 and 293 cell lines both showed strong chemo-resistance with 293 cells displaying slightly stronger chemo-resistance than PANC-1. Previous studies also revealed the same results as concluded in this study (38,39,44). PANC-1 was found to have low sensitivity to gemcitabine in a previous study (40). MIA PaCa-2 and PANC-1 are both primary tumour cells (35). The difference in the IC_{50} values between these cell lines could be due to the fact that PANC-1 cells are more resistant as compared to MIA PaCa-2 cells. Moreover, in a previous study it was reported that even at very low doses (5-10 nM), gemcitabine was able to induce nuclear factor-κB (NF-κB) activity, which can promote gemcitabine chemo-resistance (45). Hence, it can be surmised that doses (>50 nM) used in this study could significantly activate the NF-κB activity.

In the present study, fucoxanthin inhibited the viability of human pancreatic cancer cell lines, MIA PaCa-2 and PANC-1, as well as 293 cells in a dose-dependent manner. Fucoxanthin time-dependently suppressed the proliferation of MIA PaCa-2 and 293 cells. In the higher fucoxanthin concentration treatment groups of each cell line, formation of nuclear condensation was evidently clear when observed under the inverted microscope (data not shown). The antioxidant property of fucoxanthin was considered to be one of the major reasons for the anticancer effect of fucoxanthin (46-48). NF-κB activity has been demonstrated to be inhibited by antioxidants (49,50). It has been suggested that some part of fucoxanthin is hydrolysed to fucoxanthinol during the uptake (51). Fucoxanthinol is then further converted into amarouciaxanthin A in HepG2 cells. These two fucoxanthin metabolites were found to reduce the viability of human prostate cancer cell line PC-3 (27,52). Fucoxanthinol was also found to have more efficient anti-proliferative effects than fucoxanthin (23,53,54). The difference in sensitivity for different cell lines may be due to the different content of hydrolytic enzymes in the different cells.

In the present investigation, gemcitabine was used with fucoxanthin simultaneously, to explore their combined effects on pancreatic cancer cells. To the best of our knowledge, applying a fucoxanthin concentration range under 1 μM in anticancer research was performed for the first time. The aim of using low fucoxanthin concentrations was to determine whether fucoxanthin is able to effectively improve the cytotoxicity of gemcitabine even at low concentrations. The carotenoid fucoxanthin is known to sensitize multidrug-resistant cancer cells, and a proposed mechanism for fucoxanthin overcoming multiple drug resistance in cancer cells and increasing efficacy of chemotherapy in targeted cells has been reported (55).

The findings of the present study may potentially increase the benefits of fucoxanthin-coupled treatments. The combined effect of fucoxanthin and gemcitabine on the reduction of MIA PaCa-2 cell viability was significantly higher than either gemcitabine (25 and 50 nM) or fucoxanthin (150, 250 and 300 nM) used alone. A synergistic effect was only observed in the 25-nM gemcitabine combined with fucoxanthin at concentrations higher than 250-nM groups. In addition, gemcitabine (50 nM) with fucoxanthin only demonstrated an additive effect.

A possible explanation for this observation may be that gemcitabine at a concentration of 50 nM is highly cytotoxic, and therefore low doses of fucoxanthin cannot provide additional effects beyond the chemotherapeutic dose. PANC-1 cells were found to be resistant to gemcitabine and sensitive to fucoxanthin treatment. Thus, fucoxanthin (10 and 20 μM) played a leading role in the combination effect in the suppression of PANC-1 cells. Fucoxanthin was shown to significantly enhance the inhibitory effect of gemcitabine. The cell viability under the combination treatment was significantly lower than that in cells treated only with fucoxanthin or gemcitabine. However, the combination effect was not synergistically improved by these two drugs.

In the present study, it was found that low doses of fucoxanthin treatment (150, 250, 300 nM) could not inhibit the growth of 293 cells for 72 h. However, high concentrations of the fucoxanthin (10 and 20 μM) significantly suppressed the proliferation of 293 cells. Low concentrations of fucoxanthin did not promote the inhibitory effect of gemcitabine (25 nM). Interestingly, in the 50-nM gemcitabine groups, low doses of fucoxanthin aided in reducing the cytotoxicity of gemcitabine. In addition, there was more 293 cell survival under the incubation with combination treatment for 72 h in relation to cells cultured only with gemcitabine (50 nM). Fucoxanthin was demonstrated to have no inhibitory effect on human lymphocyte cells, uninfected leukaemia cell lines and human peripheral blood mononuclear cells, over a certain concentration range (23). These literature studies potentially indicate that fucoxanthin is still toxic to non-cancer cells when used at high doses, although its cytotoxicity is selective. Through its mechanism of action, gemcitabine has been determined as a pyrimidine nucleotide analog to be involved in DNA synthesis, thereby inhibiting the DNA synthesis and relating to cell division from the whole process of cell mitosis (56-59). Conversely, fucoxanthin exerts it anti-proliferative and cancer-preventing effects via different molecules and pathways including the Bcl-2 proteins, MAPK, apoptosis, or metastasis (51). Kumar *et al* reported that the anti-proliferative effects of fucoxanthin are selective, i.e., fucoxanthin has the capability to target cancer cells only, leaving normal physiological cells unaffected or less affected (51). Therefore, similar to the results of this previous study, the results of the present study indicate that fucoxanthin selectively exerts its effect only on pancreatic cancer cells.

A suitable concentration range of fucoxanthin and gemcitabine has to be established in order to create synergistic effects in cancer cell growth inhibition and neutralize toxicity to non-cancer cells. The arrest of cell cycle progression, induction of apoptosis, or both, are factors in the inhibition of cancer cell proliferation (60). The anticancer activity of gemcitabine is primarily performed by impairing DNA synthesis. It results in the cytostasis owing to the block of the cell cycle in the G_0-G_1 or S phases (61). Subsequently, cells may undergo apoptosis or mitotic catastrophe upon escaping the cell cycle blockage, which will finally lead to cell death (62). Gemcitabine, depending on its exposure time and concentration, has been demonstrated to block certain human solid tumour cells in the S phase (63). However, a previous study indicated that gemcitabine arrests cells in the G_0-G_1, G_1, early S or S phases only depending upon the concentration of gemcitabine (64). In the present study, gemcitabine was demonstrated to dose-dependently arrest the MIA PaCa-2 cells in the S phase after both 48- and 72-h exposure. Sub-G_1 is an index of apoptotic DNA fragmentation (65). The results of the present study indicated that gemcitabine first blocked MIA PaCa-2 cells in the S phase and then induced cell apoptosis. The same result was obtained in 293 cells. However, gemcitabine was revealed to arrest PANC-1 cells in the G_0-G_1 phase after culture for 48 and 72 h. Gemcitabine induced the apoptosis of PANC-1 cells in a time- and dose-dependent manner. Taken together, the results of the present study on MIA PaCa-2, PANC-1 and 293 cells are consistent with previous research aforementioned, namely that gemcitabine induces G_0-G_1 and S phase arrest and subsequently undergoes apoptosis. Moreover, all these results are consistent with the results of the cytotoxicity analysis.

Carotenoids have been demonstrated to inhibit tumour cell growth by inducing cell cycle arrest at the G_1 phase and/or apoptosis (66,67). Fucoxanthin has been suggested to accumulate cells in the G_0-G_1 phase of different cell lines as previously reported (68). In the present study, higher concentrations of fucoxanthin were found to arrest the cells in the G_0-G_1 phase. Fucoxanthin has been observed to block the human gastric adenocarcinoma cell line, MGC-803, in the G_2-M phase (69). By contrast, it has been suggested that carotenoids cannot arrest cells in the G_2-M phase (70). Thus, whether fucoxanthin induces the arrest of cells in the G_2-M phase requires further research. Low doses of fucoxanthin (150, 250 and 300 nM) could not markedly alter the cell cycle distribution. This is consistent with the cytotoxicity analysis.

Fucoxanthin combined with gemcitabine was found to help induce pancreatic cancer cell arrest in the G_0-G_1 or S phase. The lack of cell cycle arrest by fucoxanthin on 293 cells implies that fucoxanthin has selective toxicity. Both gemcitabine and fucoxanthin block cells in the G_1/S phase and the effects of each compound on cellular metabolism are different. From literature reported, carotenoids reverse multidrug resistance and enhance sensitivity of cancer treatment in *in vitro* and *in vivo* models (55,71,72). However, in the present study only additive effects were observed. A plausible explanation for this observation may be the sequence of drug administration. The administration of scheduled medications has been demonstrated to be very important for the antitumor effect. The same drugs with different treatment sequences were found to produce differing results (73). Gemcitabine and fucoxanthin were added simultaneously to the cell lines in the present study. Therefore, pre-treating the cells with one of the compounds and later treating these cells with the second one should be assessed in further studies. Nevertheless, low doses of fucoxanthin were revealed to help improve the anti-proliferative efficacy of gemcitabine by inducing growth arrest, while high doses of fucoxanthin inhibited proliferation by inducing apoptosis. The synergistic effect could be observed by using the best combination concentrations and optimal treatment sequence of fucoxanthin and gemcitabine.

In conclusion, fucoxanthin effectively improved the cytotoxicity of gemcitabine even at low concentrations, and as a type of carotenoid, fucoxanthin generated a synergistic effect increasing the sensitivity of chemotherapy in certain pancreatic cancer cell lines. Fucoxanthin also exhibited selective toxicity against cancer cells even at low concentrations. Thus, fucoxanthin, the most abundant carotenoid found in marine algae, at low therapeutic concentrations, may be considered as a potential adjunct treatment for pancreatic cancer in combination with other clinical cancer chemotherapy drugs. A limitation of the present study is identified in the MTT assay. This assay was used as a single method for cell viability determination, while other methods may also be applied. The color of fucoxanthin itself may be a potential risk that could affect the actual result of the colorimetric assay. Hence other cell viability assays such as Cell Counting Kit-8 or crystal violet assays may be used to validate the cytotoxic results. In addition, using non-cancerous pancreas-origin cell lines may be a more appropriate choice for future study. Therefore, the present results are not conclusive. Furthermore, more data should be generated, such as changes in cell morphology, and mechanism studies detailing the effect of fucoxanthin on caspase-3 or caspase-9 should be performed to demonstrate the effectiveness of fucoxanthin treatment. In addition, the selectivity of observed fucoxanthin bioactivity should be examined using *in vivo* studies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding authors.

Authors' contributions

JLu, JLi, TY, BZ and TF conceived the study. JLu, KSW, XJW, YL, LC, YZ, MJ, JLiu designed the study and acquired the data. AH, YH, JLu, XJW and KSW analyzed the data. JLu, JLi, TY, BZ and TF provided experimental materials. XJW, AH, KSW and JLu wrote the original draft. YL, LC, YZ, MJ, JLiu, JLu, JLi, TY, BZ, TF and YH revised the work critically for important intellectual content. TF, KSW and JLu confirm the

authenticity of all the raw data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. American Cancer Society: Cancer Facts and Figures. American Cancer Society, Atlanta, pp1-56, 2017.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917, 2010.
3. Neoptolemos JP, Urrutia R, Abbruzzese JL and Büchler MW: Pancreatic cancer. New York, NY, Springer, 2010.
4. Ministry of Health. Cancer programme. 2015. Retrieved March 19, 2015, from http://www.health.govt.nz/our-work/diseases-and-conditions/cancer-programme.
5. Bosetti C, Bertuccio P, Negri E, La Vecchia C, Zeegers MP and Boffetta P: Pancreatic cancer: Overview of descriptive epidemiology. Mol Carcinog 51: 3-13, 2012.
6. Goodman KA and Hajj C: Role of radiation therapy in the management of pancreatic cancer. J Surg Oncol 107: 86-96, 2013.
7. Morton JP, Timpson P, Karim SA, Ridgway RA, Athineos D, Doyle B, Jamieson NB, Oien KA, Lowy AM, Brunton VG, *et al*: Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. Proc Natl Acad Sci USA 107: 246-251, 2010.
8. Mulcahy MF, Wahl AO and Small W Jr: The current status of combined radiotherapy and chemotherapy for locally advanced or resected pancreas cancer. J Natl Compr Canc Netw 3: 637-642, 2005.
9. Peng J, Yuan JP, Wu CF and Wang JH: Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: Metabolism and bioactivities relevant to human health. Mar Drugs 9: 1806-1828, 2011.
10. Zhang H, Tang Y, Zhang Y, Zhang S, Qu J, Wang X, Kong R, Han C and Liu Z: Fucoxanthin: A promising medicinal and nutritional ingredient. Evid Based Complement Alternat Med 2015: 723515, 2015.
11. Beppu F, Niwano Y, Sato E, Kohno M, Tsukui T, Hosokawa M and Miyashita K: In vitro and in vivo evaluation of mutagenicity of fucoxanthin (FX) and its metabolite fucoxanthinol (FXOH). J Toxicol Sci 34: 693-698, 2009.
12. Zorofchian Moghadamtousi S, Karimian H, Khanabdali R, Razavi M, Firoozinia M, Zandi K and Abdul Kadir H: Anticancer and antitumor potential of fucoidan and fucoxanthin, two main metabolites isolated from brown algae. ScientificWorldJournal 2014: 768323, 2014.
13. Haefner B: Drugs from the deep: Marine natural products as drug candidates. Drug Discov Today 8: 536-544, 2003.
14. Aravindan S, Delma CR, Thirugnanasambandan SS, Herman TS and Aravindan N: Anti-pancreatic cancer deliverables from sea: First-hand evidence on the efficacy, molecular targets and mode of action for multifarious polyphenols from five different brown-algae. PLoS One 8: e61977, 2013.
15. Gammone MA and D'Orazio N: Anti-obesity activity of the marine carotenoid fucoxanthin. Mar Drugs 13: 2196-2214, 2015.
16. López-Rios L, Vega T, Chirino R, Jung JC, Davis B, Pérez-Machín R and Wiebe JC: Toxicological assessment of Xanthigen® nutraceutical extract combination: Mutagenicity, genotoxicity and oral toxicity. Toxicol Rep 9: 1021-1031, 2018.
17. Delma CR, Thirugnanasambandan S, Srinivasan GP, Raviprakash N, Manna SK, Natarajan M and Aravindan N: Fucoidan from marine brown algae attenuates pancreatic cancer progression by regulating p53-NFκB crosstalk. Phytochemistry 167: 112078, 2019.
18. Beppu F, Niwano Y, Tsukui T, Hosokawa M and Miyashita K: Single and repeated oral dose toxicity study of fucoxanthin (FX), a marine carotenoid, in mice. J Toxicol Sci 34: 501-510, 2009.
19. Hashimoto T, Ozaki Y, Mizuno M, Yoshida M, Nishitani Y, Azuma T, Komoto A, Maoka T, Tanino Y and Kanazawa K: Pharmacokinetics of fucoxanthinol in human plasma after the oral administration of kombu extract. Br J Nutr 107: 1566-1569, 2012.
20. Dang TT, Bowyer MC, Van Altena IA and Scarlett CJ: Comparison of chemical profile and antioxidant properties of the brown algae. Int J Food Sci Technol 53: 174-181, 2008.
21. Guan B, Chen K, Tong Z, Chen L, Chen Q and Su J: Advances in fucoxanthin research for the prevention and treatment of inflammation-related diseases. Nutrients 14: 4768, 2022.
22. Mumu M, Das A, Emran TB, Mitra S, Islam F, Roy A, Karim MM, Das R, Park MN, Chandran D, *et al*: Fucoxanthin: A promising phytochemical on diverse pharmacological targets. Front Pharmacol 13: 929442, 2022.
23. Ishikawa C, Tafuku S, Kadekaru T, Sawada S, Tomita M, Okudaira T, Nakazato T, Toda T, Uchihara JN, Taira N, *et al*: Anti-adult T-cell leukemia effects of brown algae fucoxanthin and its deacetylated product, fucoxanthinol. Int J Cancer 123: 2702-2712, 2008.
24. Liu CL, Liang AL and Hu ML: Protective effects of fucoxanthin against ferric nitrilotriacetate-induced oxidative stress in murine hepatic BNL CL.2 cells. Toxicol In Vitro 25: 1314-1319, 2011.
25. Liu CL, Lim YP and Hu ML: Fucoxanthin enhances cisplatin-induced cytotoxicity via NFκB-mediated pathway and downregulates DNA repair gene expression in human hepatoma HepG2 cells. Mar Drugs 11: 50-66, 2013.
26. Wang SK, Li Y, White WL and Lu J: Extracts from New Zealand *Undaria pinnatifida* containing fucoxanthin as potential functional biomaterials against cancer in vitro. J Funct Biomater 5: 29-42, 2014.
27. Martin LJ: Fucoxanthin and its metabolite fucoxanthinol in cancer prevention and treatment. Mar Drugs 13: 4784-4798, 2015.
28. Takahashi K, Hosokawa M, Kasajima H, Hatanaka K, Kudo K, Shimoyama N and Miyashita K: Anticancer effects of fucoxanthin and fucoxanthinol on colorectal cancer cell lines and colorectal cancer tissues. Oncol Lett 10: 1463-1467, 2015.
29. Terasaki M, Kubota A, Kojima H, Maeda H, Miyashita K, Kawagoe C, Mutoh M and Tanaka T: Fucoxanthin and colorectal cancer prevention. Cancers (Basel) 13: 2379, 2021.
30. Ming JX, Wang ZC, Huang Y, Ohishi H, Wu RJ, Shao Y, Wang H, Qin MY, Wu ZL, Li YY, *et al*: Fucoxanthin extracted from *Laminaria japonica* inhibits metastasis and enhances the sensitivity of lung cancer to Gefitinib. J Ethnopharmacol 265: 113302, 2021.
31. Malhão F, Macedo AC, Costa C, Rocha E and Ramos AA: Fucoxanthin holds potential to become a drug adjuvant in breast cancer treatment: Evidence from 2D and 3D cell cultures. Molecules 26: 4288, 2021.
32. Ye GL, Du DL, Jin LJ and Wang LL: Sensitization of TRAIL-resistant cervical cancer cells through combination of TRAIL and fucoxanthin treatments. Eur Rev Med Pharmacol Sci 21: 5594-5601, 2017.
33. Hidalgo M, Cascinu S, Kleeff J, Labianca R, Löhr JM, Neoptolemos J, Real FX, Van Laethem JL and Heinemann V: Addressing the challenges of pancreatic cancer: Future directions for improving outcomes. Pancreatology 15: 8-18, 2015.
34. Jiang PH, Motoo Y, Sawabu N and Minamoto T: Effect of gemcitabine on the expression of apoptosis-related genes in human pancreatic cancer cells. World J Gastroenterol 12: 1597-1602, 2006.
35. Gradiz R, Silva HC, Carvalho L, Botelho MF and Mota-Pinto A: MIA PaCa-2 and PANC-1-pancreas ductal adenocarcinoma cell lines with neuroendocrine differentiation and somatostatin receptors. Sci Rep 6: 21648, 2016.
36. Ghasemi M, Turnbull T, Sebastian S and Kempson I: The MTT assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. Int J Mol Sci 22: 12827, 2021.
37. Cho YS and Cho-Chung YS: Antisense protein kinase A RIalpha acts synergistically with hydroxycamptothecin to inhibit growth and induce apoptosis in human cancer cells: Molecular basis for combinatorial therapy. Clin Cancer Res 9: 1171-1178, 2003.

38. Bocci G, Fioravanti A, Orlandi P, Bernardini N, Collecchi P, Del Tacca M and Danesi R: Fluvastatin synergistically enhances the antiproliferative effect of gemcitabine in human pancreatic cancer MIAPaCa-2 cells. Br J Cancer 93: 319-330, 2005.
39. Yeo D, Huynh N, Beutler JA, Christophi C, Shulkes A, Baldwin GS, Nikfarjam M and He H: Glaucarubinone and gemcitabine synergistically reduce pancreatic cancer growth via down-regulation of P21-activated kinases. Cancer Lett 346: 264-272, 2014.
40. Rathos MJ, Joshi K, Khanwalkar H, Manohar SM and Joshi KS: Molecular evidence for increased antitumor activity of gemcitabine in combination with a cyclin-dependent kinase inhibitor, P276-00 in pancreatic cancers. J Transl Med 10: 161, 2012.
41. Wu S, Guo J, Wei W, Zhang J, Fang J and Beebe SJ: Enhanced breast cancer therapy with nsPEFs and low concentrations of gemcitabine. Cancer Cell Int 14: 98, 2014.
42. Kornmann M, Butzer U, Blatter J, Beger HG and Link KH: Pre-clinical evaluation of the activity of gemcitabine as a basis for regional chemotherapy of pancreatic and colorectal cancer. Eur J Surg Oncol 26: 583-587, 2000.
43. Hernández P, Olivera P, Dueñas-Gonzalez A, Pérez-Pastenes MA, Zárate A, Maldonado V and Meléndez-Zajgla J: Gemcitabine activity in cervical cancer cell lines. Cancer Chemother Pharmacol 48: 488-492, 2001.
44. Yong-Xian G, Xiao-Huan L, Fan Z and Guo-Fang T: Gemcitabine inhibits proliferation and induces apoptosis in human pancreatic cancer PANC-1 cells. J Cancer Res Ther 12 (Suppl): S1-S4, 2016.
45. Iwase R, Haruki K, Fujiwara Y, Furukawa K, Shiba H, Uwagawa T, Misawa T, Ohashi T and Yanaga K: Combination chemotherapy of nafamostat mesylate with gemcitabine for gallbladder cancer targeting nuclear factor-κB activation. J Surg Res 184: 605-612, 2013.
46. Rousseau EJ, Davison AJ and Dunn B: Protection by beta-carotene and related compounds against oxygen-mediated cytotoxicity and genotoxicity: Implications for carcinogenesis and anticarcinogenesis. Free Radic Biol Med 13: 407-433, 1992.
47. Bertram JS and Vine AL: Cancer prevention by retinoids and carotenoids: Independent action on a common target. Biochim Biophys Acta 1740: 170-178, 2005.
48. Pádua D, Rocha E, Gargiulo D and Ramos A: Bioactive compounds from brown seaweeds: Phloroglucinol, fucoxanthin and fucoidan as promising therapeutic agents against breast cancer. Phytochem Lett 14: 91-98, 2015.
49. Lee SJ, Bai SK, Lee KS, Namkoong S, Na HJ, Ha KS, Han JA, Yim SV, Chang K, Kwon YG, *et al*: Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing I(kappa)B kinase-dependent NF-kappaB activation. Mol Cells 16: 97-105, 2003.
50. Campo GM, Avenoso A, Campo S, D'Ascola A, Traina P, Samà D and Calatroni A: The antioxidant effect exerted by TGF-1beta-stimulated hyaluronan production reduced NF-kB activation and apoptosis in human fibroblasts exposed to FeSo4 plus ascorbate. Mol Cell Biochem 311: 167-177, 2008.
51. Kumar SR, Hosokawa M and Miyashita K: Fucoxanthin: A marine carotenoid exerting anti-cancer effects by affecting multiple mechanisms. Mar Drugs 11: 5130-5147, 2013.
52. Fan M, Nath AK, Tang Y, Choi YJ, Debnath T, Choi EJ and Kim EK: Investigation of the anti-prostate cancer properties of marine-derived compounds. Mar Drugs 16: 160, 2018.
53. Asai A, Sugawara T, Ono H and Nagao A: Biotransformation of fucoxanthinol into amarouciaxanthin A in mice and HepG2 cells: Formation and cytotoxicity of fucoxanthin metabolites. Drug Metab Dispos 32: 205-211, 2004.
54. Maeda H, Hosokawa M, Sashima T, Takahashi N, Kawada T and Miyashita K: Fucoxanthin and its metabolite, fucoxanthinol, suppress adipocyte differentiation in 3T3-L1 cells. Int J Mol Med 18: 147-152, 2006.
55. Eid SY, Althubiti MA, Abdallah ME, Wink M and El-Readi MZ: The carotenoid fucoxanthin can sensitize multidrug resistant cancer cells to doxorubicin via induction of apoptosis, inhibition of multidrug resistance proteins and metabolic enzymes. Phytomedicine 77: 153280, 2020.
56. Baker CH, Banzon J, Bollinger JM, Stubbe J, Samano V, Robins MJ, Lippert B, Jarvi E and Resvick R: 2'-Deoxy-2'-methylenecytidine and 2'-deoxy-2',2'-difluorocytidine 5'-diphosphates: Potent mechanism-based inhibitors of ribonucleotide reductase. J Med Chem 34: 1879-1884, 1991.
57. Jones RM, Kotsantis P, Stewart GS, Groth P and Petermann E: BRCA2 and RAD51 promote double-strand break formation and cell death in response to gemcitabine. Mol Cancer Ther 13: 2412-2421, 2014.
58. Li Y, Wang LR, Chen J, Lou Y and Zhang GB: First-line gemcitabine plus cisplatin in nonsmall cell lung cancer patients. Dis Markers 2014: 960458, 2014.
59. Ke Z, Fu T, Wang X, Xuan M, Yin H, Zhou J, Liu Y and Liang A: CHK1 inhibition overcomes gemcitabine resistance in non-small cell lung cancer cell A549. Res Sq, 2022.
60. Du L, Lyle CS, Obey TB, Gaarde WA, Muir JA, Bennett BL and Chambers TC: Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity: Evidence that mitotic Bcl-2 phosphorylation is JNK-independent. J Biol Chem 279: 11957-11966, 2004.
61. Bildstein L, Pili B, Marsaud V, Wack S, Meneau F, Lepêtre-Mouelhi S, Desmaële D, Bourgaux C, Couvreur P and Dubernet C: Interaction of an amphiphilic squalenoyl prodrug of gemcitabine with cellular membranes. Eur J Pharm Biopharm 79: 612-620, 2011.
62. Mc Gee MM: Targeting the mitotic catastrophe signaling pathway in cancer. Mediators Inflamm 2015: 146282, 2015.
63. Montano R, Thompson R, Chung I, Hou H, Khan N and Eastman A: Sensitization of human cancer cells to gemcitabine by the Chk1 inhibitor MK-8776: Cell cycle perturbation and impact of administration schedule in vitro and in vivo. BMC Cancer 13: 604, 2013.
64. Guo JR, Chen QQ, Lam CW, Wang CY, Wong VK, Chang ZF and Zhang W: Profiling ribonucleotide and deoxyribonucleotide pools perturbed by gemcitabine in human non-small cell lung cancer cells. Sci Rep 6: 37250, 2016.
65. Kajstura M, Halicka HD, Pryjma J and Darzynkiewicz Z: Discontinuous fragmentation of nuclear DNA during apoptosis revealed by discrete 'sub-G1' peaks on DNA content histograms. Cytometry A 71: 125-131, 2007.
66. Haddad NF, Teodoro AJ, Leite de Oliveira F, Soares N, de Mattos RM, Hecht F, Dezonne RS, Vairo L, Goldenberg RC, Gomes FC, *et al*: Lycopene and beta-carotene induce growth inhibition and proapoptotic effects on ACTH-secreting pituitary adenoma cells. PLoS One 8: e62773, 2013.
67. Milani A, Basirnejad M, Shahbazi S and Bolhassani A: Carotenoids: Biochemistry, pharmacology and treatment. Br J Pharmacol 174: 1290-1324, 2017.
68. Das SK, Hashimoto T, Shimizu K, Yoshida T, Sakai T, Sowa Y, Komoto A and Kanazawa K: Fucoxanthin induces cell cycle arrest at G0/G1 phase in human colon carcinoma cells through up-regulation of p21WAF1/Cip1. Biochim Biophys Acta 1726: 328-335, 2005.
69. Yu RX, Hu XM, Xu SQ, Jiang ZJ and Yang W: Effects of fucoxanthin on proliferation and apoptosis in human gastric adenocarcinoma MGC-803 cells via JAK/STAT signal pathway. Eur J Pharmacol 657: 10-19, 2011.
70. Koklesova L, Liskova A, Samec M, Buhrmann C, Samuel SM, Varghese E, Ashrafizadeh M, Najafi M, Shakibaei M, Büsselberg D, *et al*: Carotenoids in cancer apoptosis-the road from bench to bedside and back. Cancers (Basel) 12: 2425, 2020.
71. García-Olmo DC, Riese HH, Escribano J, Ontañón J, Fernandez JA, Atiénzar M and García-Olmo D: Effects of long-term treatment of colon adenocarcinoma with crocin, a carotenoid from saffron (*Crocus sativus* L.): An experimental study in the rat. Nutr Cancer 35: 120-126, 1999.
72. Eid SY, El-Readi MZ and Wink M: Carotenoids reverse multidrug resistance in cancer cells by interfering with ABC-transporters. Phytomedicine 19: 977-987, 2012.
73. Kwon M, Jung H, Nam GH and Kim IS: The right timing, right combination, right sequence, and right delivery for cancer immunotherapy. J Control Release 331: 321-334, 2021.

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