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Myricetin Induces Pancreatic Cancer Cell Death via the Induction of Apoptosis and Inhibition of the Phosphatidylinositol 3-Kinase (PI3K) Signaling Pathway

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Abstract

Pancreatic cancer is the fourth leading cause of cancer related deaths and is a disease with poor prognosis. It is refractory to standard chemotherapeutic drugs or to novel treatment modalities, making it imperative to find new treatments. In this study, using both primary and metastatic pancreatic cancer cell lines, we have demonstrated that the flavonoid myricetin induced pancreatic cancer cell death *in vitro* via apoptosis, and caused a decrease in PI3 kinase activity. *In vivo*, treatment of orthotopic pancreatic tumors with myricetin resulted in tumor regression and decreased metastatic spread. Importantly, myricetin was non-toxic, both *in vitro* and *in vivo*, underscoring its use as a therapeutic agent against pancreatic cancer.

Keywords

Pancreatic Cancer; Myricetin; Orthotopic model; Apoptosis

1. Introduction

Pancreatic cancer is a disease associated with a dismal prognosis. It is the fourth leading cause of cancer-related deaths in the US, with a 5-year survival rate of less than 5%. Pancreatic cancer is refractory to standard chemotherapeutic drugs like gemcitabine and 5-fluorouracil. Therefore, there is an urgent need to develop effective treatments against this disease. Flavonoids are anti-cancer agents, which, when taken orally, reduce the risk for developing various cancers [1; 2; 3].

Flavonols, which include kaempferol, myricetin and quercetin, are present in a wide variety of fruits and vegetables. Interestingly, all three compounds are thought to exert their anti-cancer effects mainly via their anti-oxidant properties. We and others have recently shown that quercetin decreases pancreatic cancer growth via induction of apoptosis [4; 5]. There is accumulating evidence to suggest that flavanols can also directly modulate the activity of a

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Conflict of Interest Statement:

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large number of important pro- and anti-apoptotic signaling molecules. For example, myricetin acts as a potent inhibitor of the known anti-apoptotic signaling pathways, extracellular mitogen-activated protein kinase (ERK1/2) in lung cancer cell lines [6] and phosphatidylinositol 3-kinase (PI3-K) in cervical and lung cancer cell lines [7; 8; 9; 10; 11; 12]. These pathways, through their ability to affect growth and survival of cancer cells, lead to cell cycle progression and proliferation. However, the efficacy of myricetin as an anti-cancer agent in pancreatic cancer is unknown. Therefore, the aims of this study were to examine the effect of myricetin on i) pancreatic cancer cell growth and apoptosis *in vitro*; ii) PI3-kinase signaling cascade in pancreatic cancer cells; and iii) pancreatic cancer growth *in vivo*.

2. Materials and Methods

2.1. Reagents

Myricetin was purchased from Sigma Aldrich. Antibodies were purchased as follows: anti-mouse HSP70 (Stressgen), anti-goat-actin (Santa Cruz Biotechnology Inc.), anti-pAkt and anti-Akt (Cell Signaling). Assay kits were obtained as follows: WST-8 viability assay (Dojindo Molecular Technologies), Caspase-Glo assays (Promega), Guava Nexin Apoptosis Kit (Guava Technologies Inc.) and Cell Death Detection Kit (Roche). Cells were maintained in culture medium, Fetal Bovine Serum and Penicillin-Streptomycin from Hyclone. Bovine pituitary extract and insulin-selenium-transferrin were obtained from Invitrogen. All other reagents were from purchased from Sigma.

2.2. Cell Culture

Pancreatic cancer cells were cultured in DMEM (MIA PaCa-2 and Panc-1) or RPMI (S2-013), containing 10% FBS and 1% Penicillin-Streptomycin. Mouse pancreatic ductal cells were cultured in DMEM/F-12 medium (1:1) with 5 mg/l D-glucose, 0.1 mg/ml soybean trypsin inhibitor type I, 5 ml/l ITS, 25 µg/ml bovine pituitary extract, 5 nM 3,3',5-triiodo-L-thyronine, 1 µM dexamethasone, 1.22 mg/ml nicotinamide, 5% Nu-Serum, 100 ng/ml cholera toxin and 1% penicillin-streptomycin [13]. All cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

2.3. Treatment of Cells with Myricetin

Pancreatic cancer cells (MIA PaCa-2, Panc-1 or S2-013) or normal pancreatic ductal cells (PDCs) were exposed to myricetin (12.5–200 µM) for times indicated, in serum free medium. Cells incubated with culture medium with an equivalent amount of vehicle (DMSO) served as controls.

2.4. Determination of Cell Viability

Cell viability was determined using the Dojindo Cell Counting Kit-8. Cells were seeded onto a 96-well plate at 1×10^4 cells per well and allowed to adhere overnight. After treatment with myricetin at various concentrations for 24 hours, 10 µl of the tetrazolium substrate was added to each well of the plate. Plates were incubated at 37°C for 1 hour, after which the absorbance at 450 nm was measured. All experiments were done in triplicate and repeated at least three independent times.

2.5. Measurement of Annexin V Positive Cells

Phosphatidylserine externalization was analyzed using the Guava Nexin™ Kit by flow cytometry, as previously described [4].

2.6. Terminal Deoxynucleotide Transferase-Mediated dUTP Nick End Labelling (TUNEL) Assay for Measurement of In Situ Apoptosis

Pancreatic tumor cells were cultured on chamber slides with or without myricetin (50 and 100 μ M) for 48 hours. TUNEL assays were performed using the In Situ Cell Death Detection Kit, according to the manufacturer's instructions.

2.7. Measurement of Caspase-3 and 9 Activities

Caspase-3 and 9 activities were analyzed using the Caspase-Glo® luminescent-based assays according to the manufacturer's instructions. Cells (1×10^4) were seeded into 96-well white opaque plates and a corresponding optically clear 96-well plate, and allowed to adhere overnight. The next day, cells were treated with varying concentrations of myricetin (25–200 μ M) for 6 hours. At the end of the incubation time, 100 μ l of the appropriate Caspase-Glo reagent was added to each well containing 100 μ l of blank, negative control or treated cells in culture medium. Plates were gently mixed and incubated for 1 hour at room temperature. The luminescence was then read in a luminometer (Omega, BMG LABTECH Inc., CA). The corresponding 96-well clear plate was used to measure the number of viable cells with the CCK-8 reagent. Caspase activity was normalized to these values.

2.8. Measurement of Cell Signaling Proteins in Pancreatic Cancer Cells

To assess the mechanism by which myricetin may be acting on pancreatic cancer cells the following pathways were investigated:

2.8.1. Assessment of HSP70—The expression of HSP70 was examined in pancreatic cancer cells after they were treated for 24 hours with myricetin (25–200 μ M) using western blotting as previously described [4; 14]. Actin was used as a loading control.

2.8.2 Assessment of PI3K—Phosphorylated Akt was measured by western blotting using pAkt and Akt antibodies from Cell Signaling, and analyzed with the Odyssey Infrared imaging system (Licor Biosciences). Total Akt expression was used as a loading control.

2.9. Orthotopic Pancreatic Tumors in Nude Mice

MIA PaCa-2 or S2-013 cells were orthotopically injected into the pancreas as previously described [14]. Cell viability was assessed prior to injection. Cells were resuspended in matrigel at a concentration of 1 million cells per 10 μ l matrigel and kept on ice until injected. Female nude mice (4–6 weeks old from Charles River Laboratories) were anesthetized with ketamine/Xylazine (100mg and 10mg/kg) and the surgical procedure was carried out according to the University of Minnesota Institutional Animal Care and Use Committee. Briefly, a small left abdominal flank incision was made, and the spleen and pancreas were exteriorized. MIA PaCa-2 or S2-013 tumor cells were injected into the pancreas with a Hamilton syringe. Both layers of the abdominal wound were closed with stitches and wound clips. 7 days after tumor transplantation, mice were anesthetized and tumor size was imaged using ultrasound. This technique determined that all tumors were of a similar size. Mice were then randomized into two treatment groups: *i*) control and *ii*) myricetin. Mice were given daily intraperitoneal injections of myricetin (30mg/kg in the MIA PaCa-2 model and 50mg/kg in the S2-013 model) or vehicle (DMSO) for 35 days (MIA PaCa-2 model) or 18 days (S2-013 model). Ultrasound measurements were performed at regular intervals to monitor tumor growth. At the end of the *in vivo* experiment, tumor size was measured using calipers and tumor volume was calculated as $0.5 \times \text{length} \times \text{width} \times \text{depth}$. Loco-regional spread was determined macroscopically at autopsy as well as by a blinded pathologist. Liver function was assessed on blood plasma collected at the end of each model.

2.10. Immunohistochemistry of pancreatic tumors

Tumors from control and myricetin treated animals were cut as 5 μ m sections and antigen retrieval performed using sodium citrate buffer (Reveal decloaker, Biocare). pAkt antibody (Dako Labs) was used in a 1:10 concentration to analyze pAkt levels in the tissue. Haematoxylin (Vector labs) was used to stain nuclei.

2.11. Statistical Analysis

Values are expressed as the mean $\pm$ SEM. The significance of the difference between the control and each experimental test condition was analyzed by unpaired Student's t-test and a value of $p < 0.05$ was considered statistically significant. For the *in vivo* experiments, analysis of the effects of myricetin on loco-regional tumor spread was performed using the Wilcoxin Rank Sum test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Myricetin Decreases Pancreatic Cancer Cell Viability

The effect of myricetin on the viability of pancreatic cancer cells or normal pancreatic ductal cells was examined after incubation in medium containing myricetin at concentrations of 12.5–200 μ M for 24h. Cells incubated in the medium without myricetin served as controls. The presence of myricetin significantly reduced cell viability in all pancreatic cancer cells tested (MIA PaCa-2, Panc-1, and S2-013) in a dose-dependent manner (Figure 1A), whereas little effect was observed on the viability of normal pancreatic ductal cells (Figure 1B). Normal pancreatic ductal cell viability was only significantly reduced after incubation with the highest dose of myricetin (200 μ M).

3.2. Myricetin Treatment Increases Apoptosis in Pancreatic Cancer Cells

Cell death can occur through a number of pathways, including apoptosis, autophagy, necrosis and disruption of cell division [15]. To assess the mechanism by which myricetin induces cell death, several markers of apoptosis were analysed, including: i) Caspase activity ii) Annexin V; and iii) TUNEL.

Apoptosis, or Type I cell death occurs through an intrinsic pathway, requiring the activation of the caspase cascade. We therefore sought to investigate the mode of myricetin-induced cell death by assaying for the activity of caspase-3 and caspase-9. Incubation of MIA PaCa-2 and S2-013 in culture medium containing myricetin for 6 hours resulted in a statistically significant increase in caspase-3 and 9 activity in a dose-dependent manner, as compared to control cells (Figure 2A and 2B). Similar results were observed with Panc-1 cells (results not shown), suggesting that myricetin induces cell death via caspase activation. Phosphatidylserine externalization is one of the earliest events in apoptosis, which can be measured by Annexin V staining. Exposure of all pancreatic cancer cells (MIA PaCa-2, Panc-1 and S2-013) to myricetin for 24h resulted in a dose-dependent increase in Annexin V positive cells (Figure 2C). This result suggests that myricetin-induced cell death in pancreatic cancer cells is facilitated by apoptosis.

To confirm that myricetin induces apoptotic cell death in pancreatic cancer cells, MIA PaCa-2, and Panc-1 cells were incubated with myricetin for 24 hours and assayed for late apoptotic events by a TUNEL assay. A significant increase in TUNEL stained cells confirm that myricetin mediates cell death via an increase in apoptosis (Figure 2D).

3.3. Myricetin Does not Effect HSP70 Levels in Pancreatic Cancer Cells

Previous data from our laboratory demonstrated that the flavonoid quercetin inhibits HSP70 expression which results in pancreatic cancer cell death [4], and that siRNA-mediated knockdown of HSP70 causes cell death in pancreatic cancer *in vitro* [16]. Given the similarity in the structures of quercetin and myricetin, it was of interest to examine the effect of myricetin on HSP70 expression in pancreatic cancer cells. Myricetin (25–200 μ M) had no effect on HSP70 at the protein (Figure 3A) or mRNA level (data not shown) in MIA PaCa-2 or S2-013 cells. Similar results were obtained for Panc-1 cells (data not shown), suggesting that myricetin induces apoptosis by a mechanism other than those regulated by HSP70.

3.4. Myricetin Inhibits PI3-kinase Signalling in Pancreatic Cancer Cells

Previous studies in lung, and cervical cancer cells show that myricetin is a potent inhibitor of the PI3-kinase pathway [7; 8; 12]. We therefore assessed the ability of myricetin to inhibit this pathway. Incubation of MIA PaCa-2 and S2-013 cells with myricetin inhibited the phosphorylation of Akt (a marker of PI3-kinase activity; Figure 3B). Our results suggest that myricetin induces apoptosis via the inhibition of the PI-3 kinase signalling pathway, but not via the HSP70 pathway.

3.5. Myricetin Inhibits Tumor Growth in an Orthotopic Mouse Model of Pancreatic Cancer

To examine the effects of myricetin on pancreatic cancer growth and local regional spread/metastasis *in vivo*, we tested the efficacy of this compound on an orthotopic model of pancreatic cancer. Human pancreatic cells MIA PaCa-2, derived from a primary pancreatic tumour, or S2-013, derived from liver metastases, were injected into the pancreas of nude mice. To determine that all tumors were of a similar size, tumors were visualized by ultrasound prior to the commencement of myricetin treatment. Mice were then randomized into two treatment groups with the same mean tumor size: i) control, and ii) myricetin. Myricetin was injected intraperitoneally daily and the experiment ended when the tumor size in control DMSO-treated mice reached termination point. Myricetin treated mice showed significantly reduced tumor growth in both the MIA PaCa-2 (Figure 4A and 4B) and S2-013 (Figure 4C) –induced tumors, as compared to controls injected with vehicle (DMSO). In support of the results obtained *in vitro* showing that myricetin decreases pAkt levels in cell lines, myricetin-treated tumors derived from S2-013 lines also show a decrease in pAkt levels compared to tumors from control animals (Fig. 4D). Tumors were measured by ultrasound prior to sacrifice and by callipers post sacrifice, with similar results. Importantly, there was a significant difference in the spread of tumors to the liver and kidney ($p=0.0235$) between the myricetin-treated and control group, but not in the spread of tumor to the lung ($p=0.4279$) in the MIA PaCa-2 model (Table I). Intriguingly, in the S2-013 model, myricetin decreased spread of the tumor to the lung ($p=0.0171$), but not to the liver or kidney ($p=0.8927$ and $p=0.2943$, respectively) (Table II). Pathology results from the MIA PaCa-2 model demonstrated that 80% of mice from the control group had local tumor spread, as compared with 57% of mice from the myricetin group. Similar results were observed in the S2-013 model with 100% of mice in the control group having local tumor spread versus 54.5% of mice in the myricetin treated group. Importantly, mice treated with myricetin at either 30mg/kg/day (MIA PaCa-2-induced tumors) and 50mg/kg/day (S2-013-induced tumors) displayed no signs of toxicity as assessed by examination of liver function (Table III and Table IV).

4. Discussion

Pancreatic cancer is refractory to standard chemotherapeutic drugs like Gemcitabine and 5-fluorouracil as well as to novel treatment modalities, and is therefore correlated with poor prognosis. Resistance to apoptosis is a key factor preventing response to therapies in

pancreatic cancer. This study has shown that myricetin induces pancreatic cancer cell death by induction of apoptosis via the inhibition of the PI3 kinase pathway, as measured by a decrease in pAkt levels. In addition, *in vivo* administration of myricetin decreased tumor growth and spread in two orthotopic mouse models of pancreatic cancer (MIA PaCa-2 and S2-013), proposing myricetin as a powerful candidate as a potential therapy against pancreatic cancer.

Myricetin has been shown to possess cytotoxic effects on several cancer cells including colon [17; 18], esophageal [19], breast [20], leukemia [21; 22], and melanoma [23] *in vitro*. However, this is the first study to examine the effect of myricetin on pancreatic cancer cell growth both *in vitro* and *in vivo*. The findings in this manuscript show that myricetin significantly decreased pancreatic cancer cell viability in three pancreatic cancer cell lines of varying aggressiveness (Fig. 1). Importantly, though myricetin was a potent inducer of pancreatic cancer cell death, it did not affect the viability of normal pancreatic ductal cells (Fig. 1). Myricetin-induced pancreatic cancer cell death was mediated by an induction of apoptosis (as assessed by increased Annexin V and TUNEL) (Fig. 2). Our findings are supported by several studies in the literature with cancer cell lines including esophageal [19], colon [18] and leukemia [22]. We further investigated the mechanism by which myricetin induced pancreatic cancer cell apoptosis by analysis of the intrinsic or mitochondrial pathway, which causes release of cytochrome c from the mitochondria and activation of caspase-9 followed by caspase-3 [24; 25]. Our results indicated that myricetin increased both caspase-9 and caspase-3 levels in pancreatic cancer cells providing evidence that the mitochondrial apoptosis pathway plays a critical role in myricetin-induced cell death. These results concur with those obtained in myricetin-treated leukemia cells [22; 26],

Although several molecules and pathways have been proposed for mediating myricetin's cytotoxic effects on cancer cells, no previous studies have examined such mechanisms in pancreatic cancer cells. We have previously demonstrated that the flavonoid quercetin and the diterpenoid triepoxide, triptolide, are potent inhibitors of heat shock protein 70 (HSP70) and cause pancreatic cancer cell death via an induction of apoptosis [4; 14]. Quercetin, upon the addition of a hydroxyl group at position 5' in the B ring, gives rise to myricetin. Therefore, we next examined whether myricetin-induced apoptosis in pancreatic cancer cells could be mediated by inhibition of HSP70. Myricetin has recently been shown to act as a HSP70 inhibitor, through its ability to decrease HSP70 ATPase activity *in vitro* and in HeLa cells [12; 27]. Our results show that myricetin had no effect on HSP70 levels in pancreatic cancer cells, suggesting that the additional hydroxyl group in myricetin retains the ATPase inhibitory activity, but prevents loss of protein (Fig. 3). Previous research has shown that myricetin levels can be measured in the plasma of rats after treatment with compounds such as rattan tea [28]. While it is important to study the physiologically relevant levels of myricetin, we believe that the inability of myricetin to affect normal cells (Fig. 1B), and most importantly, the toxicity studies on the myricetin treated animals (Tables III and IV), reveal that the doses used for our study, although sufficient to cause a significant decrease in tumor burden, are not toxic to the animal.

The PI3-kinase/Akt signaling pathway is frequently over-activated in pancreatic cancers and is believed to contribute to their aggressive phenotype and resistance to chemotherapy. Since myricetin is known to act as a potent inhibitor of the PI3 kinase pathway [8; 11], which is essential for Akt activation and subsequent cell survival, we assessed the effect of myricetin on the phosphorylation of Akt. Our results confirmed previous studies in epidermoid carcinoma cells that myricetin acts via inhibition of PI3-kinase [8]. Our data is supported by studies in pancreatic cancer cell lines where PI3-kinase inhibition induces apoptotic cell death [29]. Myricetin's therapeutic potential in pancreatic cancer was tested in two orthotopic mouse models of pancreatic cancer (MIA PaCa-2 and S2-013). Daily

administration of myricetin decreased pancreatic tumor growth *in vivo*, as well as loco-regional tumor spread. Importantly, myricetin was also found to be non-toxic, thereby highlighting its potential as a therapeutic agent in pancreatic cancer to induce apoptosis by inhibition of the PI3-kinase signaling pathway. Additionally, *in vivo* analysis of tumors obtained from both control and myricetin treated animals show a myricetin-induced decrease in pAkt levels. A previous study by Teranishi et al [30] supports the role of PI3-kinase signalling in pancreatic cancer, wherein, wortmannin, a PI3-kinase inhibitor, decreased metastasis in a pancreatic cancer model. Furthermore, Fujiwara et al. [31] found that inhibition of PI3K/Akt with LY294002 enhanced the anti-tumour activity of cisplatin in pancreatic cancer cells *in vitro* and *in vivo*.

In conclusion, myricetin causes pancreatic cancer cell death *in vitro* and *in vivo* by induction of apoptosis and its mechanism of action is via the inhibition of the PI-3 kinase pathway. Therefore, myricetin is a potential therapeutic agent that can be used to prevent the progression and metastases of pancreatic cancer.

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Figure 1. Effect of myricetin on pancreatic cancer cell (A) or normal pancreatic ductal cell (B) viability. Myricetin significantly decreased MIA PaCa-2, Panc-1 and S2-013 cell viability 24 hours post-treatment, compared to untreated controls (n=5, *p<0.001). In contrast, myricetin had no effect on normal pancreatic ductal cell viability (n=3, *p<0.05) at concentrations ≤ 100μM.

2A

2B

Figure 2.

Myricetin induces apoptosis in pancreatic cancer cells. (A) Incubation of MIA PaCa-2 and S2-013 cells with myricetin for 6 hours significantly increased caspase-3 activity compared to controls. Values represent the means $\pm$ SE ($n=3$, $p<0.05$). (B) Incubation of MIA PaCa-2 and S2-013 cells with myricetin for 6 hours significantly increased caspase-9 activity compared to controls ($n=3$, $p<0.05$). (C) MIA PaCa-2, Panc-1 or S2-013 cells were incubated for 24 hours with myricetin (25–200µM) and Annexin V levels were assessed by flow cytometry. Myricetin dose dependently increased Annexin V in all three cell lines. Values represent means $\pm$ SE ($n=3$, $*p<0.05$). (D) Cells incubated for 48 hours with myricetin were examined for *in situ* TUNEL. Both MIA PaCa-2 and Panc-1 cells treated

with myricetin had increased TUNEL positive cells compared to control cells incubated with vehicle alone.

Figure 3.

Effect of myricetin on signaling proteins in pancreatic cancer cells. (A) Myricetin does not decrease HSP70 protein levels. MIA PaCa-2 or S2-013 cells were treated with myricetin (25–200 μ M) for 24 hours. Total actin was used as a loading control (n=3). (B) Myricetin inhibits PI3-kinase signalling as measured by pAkt levels in pancreatic cancer cells. Activated Akt levels were lower in cells treated with myricetin (25–100 μ M) for 24 hours. Total Akt was used as a loading control (n=3).

4A MIA PaCa-2 Orthotopic Model **4B**

4C S2-013 Orthotopic Model

4D

Figure 4.

Myricetin inhibits pancreatic tumor growth *in vivo*. (A) Mice with orthotopic MIA PaCa-2 tumors were administered myricetin (30mg/kg) daily for 35 days. Tumor growth was measured weekly by ultrasound and with callipers at the time of sacrifice. Mice displayed significantly reduced tumor size after treatment with myricetin compared to controls (injected with vehicle alone) ($n = 5$ control and $n=7$ animals treated with myricetin, $*p<0.01$ and $*p<0.02$). (B) Representative images showing a significant reduction in tumor size at the end of 42 days post-tumor induction in tumors treated with myricetin (30mg/kg) compared to controls. (C) Mice with orthotopic S2-013 tumors display a significant decrease in tumor size after treatment with myricetin (50mg/kg) daily for 18 days compared to controls

(injected with vehicle alone) (n=10 control and n=11 animals treated with myricetin, *p<0.003 and *p<0.008). (D) Tumors from control and myricetin treated animals were analyzed for pAkt levels by immunohistochemistry. Representative images of pAkt stained sections, and controls with secondary antibody alone are shown. Nuclei were stained with haematoxylin.

Table I

Effect of myricetin on loco-regional tumor spread in the MIA PaCa-2 model of pancreatic cancer. Table shows a reduction in the number of loco-regional metastases in orthotopically growing MIA PaCa-2 tumors treated with myricetin (30mg/kg) for 35 days compared to controls (vehicle alone) (n=5 control and n=7 myricetin treatment).

Organ	Control	Myricetin	p-value
Lung	4/5	4/7	0.4279
Liver	3/5	0/7	0.0235
Kidney	3/5	0/7	0.0235
Total number of organs invaded	10	4	

Table II

Effect of myricetin on loco-regional tumor spread in the S2-013 model of pancreatic cancer. Table shows a reduction in the number of local-regional metastases in orthotopically growing S2-013 tumors treated with myricetin (50mg/kg) for 18 consecutive days compared to controls (vehicle alone) (n=10 control and n=11 myricetin treatment).

Organ	Control	Myricetin	p-value
Lung	10/10	6/11	0.0171
Liver	3/10	3/11	0.8927
Kidney	1/10	0/11	0.2943
Total number of organs invaded	14	9	

Table III

Effect of myricetin on toxicity *in vivo* in the MIA PaCa-2 model of pancreatic cancer. Table shows there was no significant difference in the toxicity profiles of mice with orthotopic MIA PaCa-2 tumors after 35 consecutive days of treatment with myricetin (30mg/kg) when compared to controls (treated with vehicle alone).

	Control (n=5)	Myricetin 30mg/kg/day (n=7)	Reference range
AST	350 ± 62.48	229.1 ± 67.84 (p=0.23)	69 – 191 IU/l
ALT	58 ± 26.95	31.6 ± 10.21 (p=0.35)	26 – 120 IU/l
Alk. Ptase	47 ± 8.74	56.6 ± 19.04 (p=0.68)	44 – 118 IU/l
T Bilirubin	0.1 ± 0.0	0.125 ± 0.025 (p=0.44)	0.3 – 0.8 mg/dl
Cholesterol	49 ± 1.87	65.8 ± 7.68 (p=0.08)	63 – 174 mg/dl
Urea N	31 ± 4.84	28 ± 3.74 (p=0.64)	19 – 34 mg/dl
Creatinine	0.1 ± 0.0	0.1 ± 0.0 (p=0.99)	0.5 – 0.8 mg/dl

Table IV

Effect of myricetin on toxicity *in vivo* in the S2-013 model of pancreatic cancer. Table shows there was no significant difference in the toxicity profiles of mice with orthotopic S2-013 tumors after 18 consecutive days of treatment with myricetin (50mg/kg) when compared to controls (treated with vehicle alone).

	Control (n=10)	Myricetin 50mg/kg/day (n=11)	Reference range
AST	132.7 ± 20.63	211.8 ± 33.94 (p=0.06)	69 – 191 IU/l
ALT	41.8 ± 14.11	42.78 ± 14.52 (p=0.96)	26 – 120 IU/l
Alk. Ptase	50.9 ± 5.11	55.56 ± 16.24 (p=0.79)	44 – 118 IU/l
T Bilirubin	0.1 ± 0.0	0.456 ± 0.343 (p=0.29)	0.3 – 0.8 mg/dl
Cholesterol	131.7 ± 6.94	127.8 ± 6.753 (p=0.69)	63 – 174 mg/dl
Urea N	19.1 ± 1.15	21.89 ± 1.14 (p=0.11)	19 – 34 mg/dl
Creatinine	0.64 ± 0.54	0.1 ± 0.0 (p=0.36)	0.5 – 0.8 mg/dl