The flavonoid quercetin inhibits pancreatic cancer growth *in vitro* and *in vivo*

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Abstract

Objectives—The flavonoid quercetin holds promise as an anti-tumor agent in several preclinical animal models. However, the efficacy of oral administration of quercetin in a pancreatic cancer mouse model is unknown.

Methods—The anti-proliferative effects of quercetin alone or in combination with gemcitabine were tested in two human pancreatic cancer cell lines using cell count and MTT assays. Apoptosis was evaluated by flow cytometry. Tumor growth *in vivo* was investigated in an orthotopic pancreatic cancer animal model using bioluminescence. Quercetin was administered orally in the diet.

Results—Quercetin inhibited the growth of pancreatic cancer cell lines, which was caused by an induction of apoptosis. In addition, dietary supplementation of quercetin attenuated the growth of orthotopically transplanted pancreatic xenografts. The combination of gemcitabine and quercetin had no additional effect compared to quercetin alone. *In vivo* quercetin caused significant apoptosis and reduced tumor cell proliferation.

Conclusions—Our data provide evidence that oral administration of quercetin was capable of inhibiting growth of orthotopic pancreatic tumors in a nude mouse model. These data suggest a possible benefit of quercetin in patients with pancreatic cancer.

Keywords

Pancreatic cancer; apoptosis; quercetin; orthotopic animal model; bioluminescence

Introduction

Pancreatic cancer is the 4th leading cause for cancer death in men and women with an estimated 43,140 estimated new cases in the United States in 2010.[1] The overall five-year survival rate is less than 5% and effective therapies are largely ineffective. Gemcitabine is the standard chemotherapeutic drug in patients with pancreatic cancer, but unfortunately it

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extends survival just a few weeks.[2] For the few patients with localized disease, surgery offers a five-year survival rate of about 35% in specialized centers.[3] In the light of the dismal statistical figures, the need for additional, efficacious treatment options is clear.

Naturally occurring compounds are commonly regarded as very intriguing agents to test for cancer prevention and therapy, mainly because of the presumed multimodal actions and low toxicity profiles.[4] In addition, combination regimens of naturally occurring compounds with standard chemo-therapeutic drugs seem very promising in providing additive or synergistic efficacy or by inducing chemo-sensitization.[5] Among various naturally occurring compounds, flavonoids are considered promising chemo-preventive and -therapeutic agents against various human cancers.[6] In epidemiological studies, diets rich in vegetables and fruits have been found to prevent the formation of cancer.[7–9] Quercetin $(3,3',4',5,7$-pentahydroxyflavone), a naturally occurring flavonol, is one of the most abundant flavonoids found in many fruits and vegetables, such as broccoli, yellow onions, and apples.[10] Quercetin has been shown to exert anti-cancer and anti-inflammatory effects through the inhibition of several intracellular pathways.[10] It has been reported that quercetin inhibits several key signaling components in cancer cells, including PI3K/Akt/mTOR, GSK-3β, NFκB, and heat shock protein 70 (HSP70).[11–15] Quercetin elicits anti-tumor effects by inducing apoptosis, autophagy, and cell cycle arrest, blocking cell migration, and inhibiting fatty acid synthesis required for *de novo* membrane synthesis.[14,16–19] More recently, quercetin has been shown to inhibit self-renewal capacity of putative pancreatic cancer stem cells.[20] In other studies quercetin suppressed local and distant tumor growth and prolonged survival in murine pancreatic cancer models.[17,21] However, the efficacy of oral administration of quercetin to suppress pancreatic cancer growth has not been investigated. This is in particular important as the bioavailability and intestinal absorption of orally administered polyphenolic compounds, e.g. flavonoids, are often limited.[22]

In this study we investigated the therapeutic potential of quercetin alone and in combination with gemcitabine, the current standard chemotherapeutic drug, in cell culture models and an orthotopic animal model using bioluminescence imaging. We found that quercetin robustly induced cell death leading to a decrease in cell growth in pancreatic cancer cells *in vitro* and if given orally significantly attenuated tumor growth *in vivo*. Importantly, the addition of quercetin to gemcitabine did not result in an enhanced anti-tumor effect compared to quercetin alone.

Material and Methods

Reagents

Quercetin dihydrate was from Sigma (St. Louis, MO, USA), and Gemcitabine HCL from Eli Lilly (Indianapolis, IN, USA). The human pancreatic cancer cell lines MIA PaCa-2 and BxPC-3 were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and were cultured as previously described.[23]

Cell growth assays

Cells were treated for 48 hours with the indicated concentration of gemcitabine, quercetin or the vehicle solution. Inhibition of cell growth was determined by cell counts and MTT according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN) and described previously.[23]

Flow Cytometry

Cells were stained with the Annexin-V-FLUOS Staining Kit (Roche) according to the manufacturer's instructions, and analyzed by flow cytometry.[24]

Orthotopic xenograft mouse model

Animal studies were approved by the Chancellor's Animal Research Committee of UCLA, in accordance with the NIH Guide for the Care and Use of Laboratory Animals. MIA PaCa-2 cells were transduced with non-replicating lentiviral vectors expressing firefly luciferase. The orthotopic xenograft model in nude mice was performed as described earlier.[25] Mice were fed the AIN-93G purified rodent diet (Dyets Inc., Bethlehem, PA) and divided in four groups of six animals each: control, gemcitabine, quercetin 1% and quercetin 1% plus gemcitabine. Quercetin was administered orally in the diet. Gemcitabine (120 mg/kg) was given i.p. at day 10, 17, 24, 31, and 38. The concentration of gemcitabine typically used in mouse studies ranges from 50–150 mg/kg.[34] Bioluminescence imaging of tumors was performed at day 4, 7, 10, 17, 24, 31, and 38. After 42 days all animals were sacrificed. Bioluminescence imaging was performed on an IVIS™ 100 Imaging System with Living Image® 2.50.1 software.

High Performance Liquid Chromatography

The stability of quercetin in the diet at room temperature was tested over 7 days using high performance liquid chromatography (HPLC). Samples were extracted with methanol and organic phases collected. An aliquot was analyzed with a RP-18 Luna column (150 × 4.6 mm, 3 µm, Phenomenex) on an Agilent 1100 HPLC system with spectrophotometric diode array. The chromatograms are recorded at 370 nm and column temperature was held at 30°C. Data were analyzed with the Hewlett Packard Chemstation® software. Concentrations of quercetin were determined by HPLC using external calibration from serial dilution of the stock solution.

Immunohistochemistry

Immunohistochemical detection of BrdU was performed with the BrdU In-Situ Detection Kit (BD Pharmingen, San Jose, CA), and TUNEL with the In Situ Cell Death Detection Kit (Roche), according to the manufacturer's instructions. Sections were examined at 40-fold magnification and BrdU and TUNEL staining expressed as percentage of positive nuclei per high power field.

Statistical Methods

Comparisons of two groups were made by t-test. Comparisons of several groups were made by a One Way Analysis of Variance (ANOVA) with post-hoc Holm-Sidak analysis for pairwise comparisons in SigmaStat 3.1 (Systat Software, Inc.). Comparisons of tumor volume and tumor weight at harvest were compared by a linear regression analysis adjusting for baseline weight. A linear mixed effects regression model with random intercept was used to compare light emission between groups over time, adjusting for baseline weight. Natural logarithmic transformation was applied to light emission to improve its normality for statistical analysis. Difference in slope (increasing rate of light emission) between each treatment group and the control group was tested. Bonferroni's adjustment was made to control the overall Type I error rate at 0.05 for each experiment. Thus, p-values smaller than $0.05/k$ are considered as statistically significant, where k is the total number of tests performed for the experiment.

Results

Quercetin inhibits cell proliferation *in vitro*

To study the effect of quercetin on the growth of pancreatic cancer cells, the undifferentiated cell line MIA PaCa-2 and moderately differentiated cell lines BxPC-3 were used. Both cell lines were treated with gemcitabine (0–10 µg/ml) or quercetin (0–75 µM) and the number of

cells counted after 48 hours. In both cell lines cell numbers were reduced at quercetin concentrations as low as 10 μM (p>0.01) and gemcitabine concentrations as low as 0.001 μg/ml (Figure 1A). To exclude the generation of hydrogen peroxide by quercetin in cell culture media as the reason for cell growth inhibition, catalase was added to quercetin (75 μM) in separate experiments. In both cell lines the addition of catalase did not prevent the growth-inhibitory effect of quercetin, suggesting that the effect of quercetin is not due to the generation of hydrogen peroxide in the media (Figure 1A, inserts). Cell growth inhibition by quercetin and gemcitabine was confirmed by MTT assays (Figure 1B). To investigate whether the addition of quercetin to gemcitabine, the current drug of choice in the treatment of pancreatic cancer in patients, has an additive or even synergistic growth inhibitory effect, MIA PaCa-2 and BxPC-3 cells were treated with gemcitabine alone, quercetin alone, or gemcitabine and quercetin combined for 48 hours. Cell counts showed that the addition of quercetin to gemcitabine further reduced the number of cells compared to gemcitabine alone (Figure 1C).

Quercetin induces apoptosis *in vitro*

Having demonstrated that quercetin treatment was associated with reduced cell growth of two pancreatic cancer cell lines, we then sought to determine the underlying mechanisms. To investigate whether quercetin alters the cell cycle we used flow cytometry and examined the distribution of MIA PaCa-2 and BxPC-3 cells in different stages of the cell cycle. Cytometric analysis showed that quercetin did not have an effect on cell cycle distribution (data not shown). To investigate the effect of quercetin on cell death, we analyzed cells by flow cytometry after annexin V and propidium iodide staining. Consistent with the inhibition of cell growth, quercetin at 10 μM increased the fraction of apoptotic cells in both cell lines (p<0.05) (Figure 2). Using this methodology apoptotic cells, positive for Annexin V and negative for propidium iodide, cluster in the lower right quadrant of the blot.

Oral administration of quercetin inhibits orthotopic tumor growth and induces tumor cell apoptosis

To study the effect of orally administered quercetin *in vivo* we used an orthotopic tumor model. Pancreatic cancer cells were stably transfected with lentiviruses encoding for luciferase to allow bioluminescent imaging in live animals. To demonstrate stable light emission over time and a linear relationship between cell number and light emission, transfected cells were first plated at 1:1, 1:2, 1:4, and 1:8 dilutions. After the addition of luciferin, light emission was stable and decreased linearly with decreasing cell numbers (Figure 3A). Furthermore, to confirm the stability of quercetin in the mouse diet, quercetin levels in the diet were measured over a period of seven days at room temperature by HPLC and were found to be stable during that time period (Figure 3B). To monitor tumor growth *in vivo* we performed bioluminescence imaging of the orthotopic tumors. We found that tumors in mice fed quercetin-supplemented diet demonstrated a significantly reduced light emission over time (Figure 3C, left panel) compared to control diet-fed animals (p=0.001), suggesting tumor growth inhibiting effects of quercetin. This effect was seen early (at day seven) and resulted in smaller tumors at harvest after 42 days (p=0.044 for tumor volume, p=0.032 for tumor weight). Animals treated with gemcitabine alone also had smaller tumors compared to control-fed mice, however, this difference did not reach significance. Combination treatment of gemcitabine with quercetin resulted in significantly reduced light emission over time (p=0.010) and smaller tumors compared to controls (p=0.032 for tumor volume, p=0.033 for tumor weight), however this effect was not significantly different than quercetin alone (Figure 3C, right panel). Quercetin levels were detected in the plasma and tumor tissues by HPLC at 8.68±1.21 μM and 2.64±1.15 nmol/g, respectively.[26] To evaluate potential mechanisms of tumor growth inhibition by quercetin, tumors were stained for BrdU (as a marker for proliferation) and TUNEL (as a marker for apoptotic cell death).

Gemcitabine alone had no significant effect on tumor cell proliferation. The addition of quercetin to gemcitabine decreased tumor cell proliferation by over 50% (Figure 4A). The effect of quercetin alone was as potent as the combination of gemcitabine and quercetin (Figure 4A). Furthermore, in accordance with our *in vitro* results we found an increase in apoptotic cell death in the tumors of gemcitabine-treated and quercetin-fed animals (Figure 4B). Again, the combination of gemcitabine and quercetin had no additional effect than quercetin alone.

Discussion

The current study provides first evidence that dietary supplementation of quercetin is effective in inhibiting pancreatic cancer growth *in vivo*. Previously, quercetin has been demonstrated to show anti-tumor efficacy in pancreatic cancer animal models.[17,21] However, in both studies quercetin was administered as intraperitoneal injections. Given the concerns about bioavailability of polyphenolic compounds, e.g. quercetin, our data provide first re-assurance that orally administered quercetin is efficacious in attenuating pancreatic cancer growth *in vivo*. Quercetin naturally occurs in the form of quercetin glucosides.[27] Quercetin glucosides can be absorbed in the intestine after luminal hydrolysis by lactase phlorizin hydrolase and subsequent diffusion of released quercetin aglycones and/or by active transport via the sodium-dependent glucose transporter with subsequent deglycosylation within the enterocyte by cytosolic β-glucosidase.[27] The quercetin aglycone is then further converted into several metabolites, including glucuronide and sulfate conjugates with or without methylation on the catechol group.[27] Although we fed the mice quercetin aglycones (instead of quercetin glucosides), HPLC analyses clearly demonstrated detectable levels of quercetin in the blood and tumor tissues,[26] indicating sufficient intestinal absorption of quercetin aglycones to attenuate orthotopic pancreatic tumor growth. In fact, it has been suggested that intestinal absorption of quercetin is favored when in the aglycone form.[28] Importantly, while normally plasma concentration of quercetin in humans is in the low nano-molar range, it was reported to be enhanced to low micromolar levels after supplementation (1g of quercetin per day),[28] which is in the same concentration range as in our animal study.[26] In pilot studies using up to 10% quercetin in the diet for eight weeks we found no overt clinical signs of toxicity as evaluated by gross daily examination of the animals and weekly body weight measurements (not shown). No change in body weight compared to control animals was noted. In addition, laboratory tests for liver and kidney functions were normal (not shown), indicating that dietary supplementation of quercetin up to 10% is well tolerated in mice. However, plasma levels of quercetin plateaued at 1% supplementation in the diet, suggesting that intestinal absorption could not be further increased.[26] For that reason, we decided to initiate a therapeutic mouse study using an experimental diet with 1% quercetin.

Interestingly, our data show that although gemcitabine, the currently approved chemotherapeutic drug in patients with pancreatic cancer, also inhibits tumor growth *in vivo* (at a similar concentration that has been used in previous reports), the combination of gemcitabine with quercetin had no additional benefit. In our animal study quercetin alone inhibited tumor growth to such an extent that could not be further enhanced by the addition of gemcitabine. This finding may simply generally reflect the well-known poor efficacy of gemcitabine in inhibiting pancreatic cancer growth in humans. Despite being the chemo-therapeutic drug of choice the response rates and improvement in overall survival with gemcitabine in patients with pancreatic cancer are marginal at best and generally disappointing so far.[29] However, more detailed and carefully designed dose-escalation studies are necessary to investigate a potential benefit in combining gemcitabine with quercetin in preclinical animal models.

Our results demonstrate that the growth inhibitory effects of quercetin are mediated by an increase in apoptotic cell death. The pro-apoptotic effect of quercetin is in accordance to other published reports in other cancer models.[30] Several mechanisms of quercetin-induced apoptosis have been implicated and may depend on the cell type studied. The more commonly discussed mechanisms include activation of AMPK, inactivation of Akt and NF-κB.[14,31,32] Since it has been suggested that the anti-proliferative action of quercetin in cell culture models is mediated by the formation of hydrogen peroxide through the interaction of the phenolic compounds with the cell culture media,[33] we tested the anti-growth properties of quercetin in pancreatic cancer cells in the presence of catalase, which degrades hydrogen peroxides. Our data clearly showed that the anti-proliferative effect of quercetin in our cell culture models was not due to the generation of hydrogen peroxides.

In summary, we demonstrated that quercetin effectively inhibits the growth of pancreatic cancer cells *in vitro* and *in vivo*. Our results suggest that the inhibitory effects are in part due to the induction of apoptosis. Furthermore, the orthotopic xenograft model provides evidence that oral administration of quercetin achieves biologically active tissue levels. Currently there is a consensus from epidemiological and human studies, that the dietary intake of polyphenols is related to a low cancer risk. These phytochemicals have been shown to be effective in several cancer models with the advantage of a low toxicity profile. Because of this favorable side-effect profile and the efficient inhibition of tumor growth shown in this preclinical model, quercetin should be further considered as a potentially adjunct treatment for pancreatic cancer.

Acknowledgments

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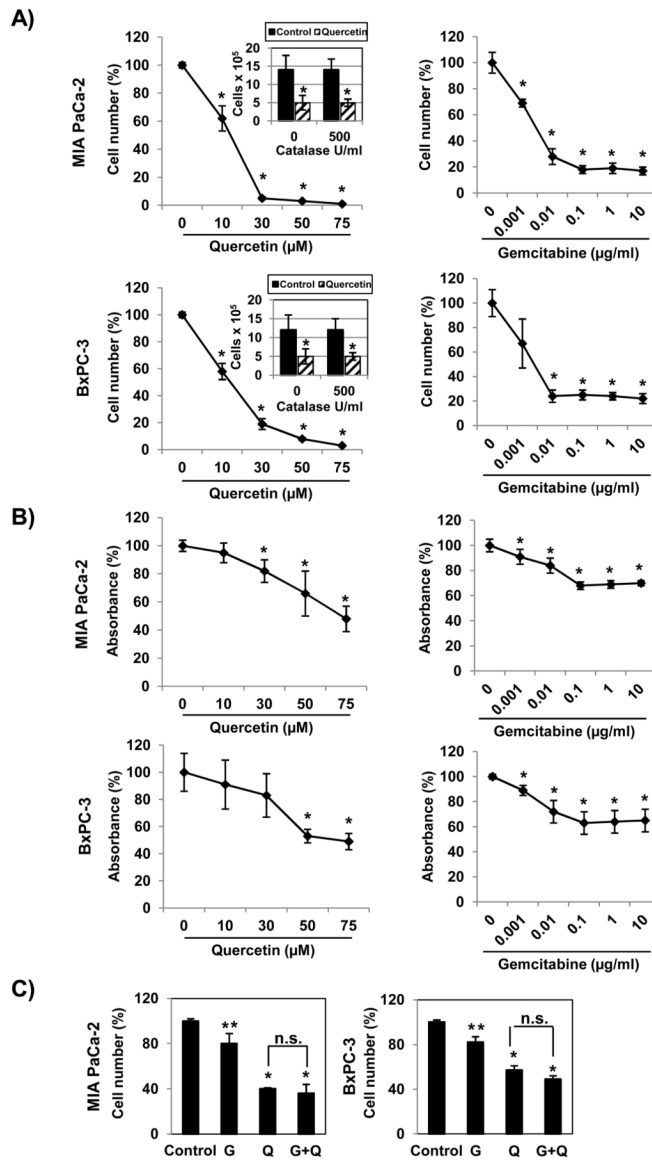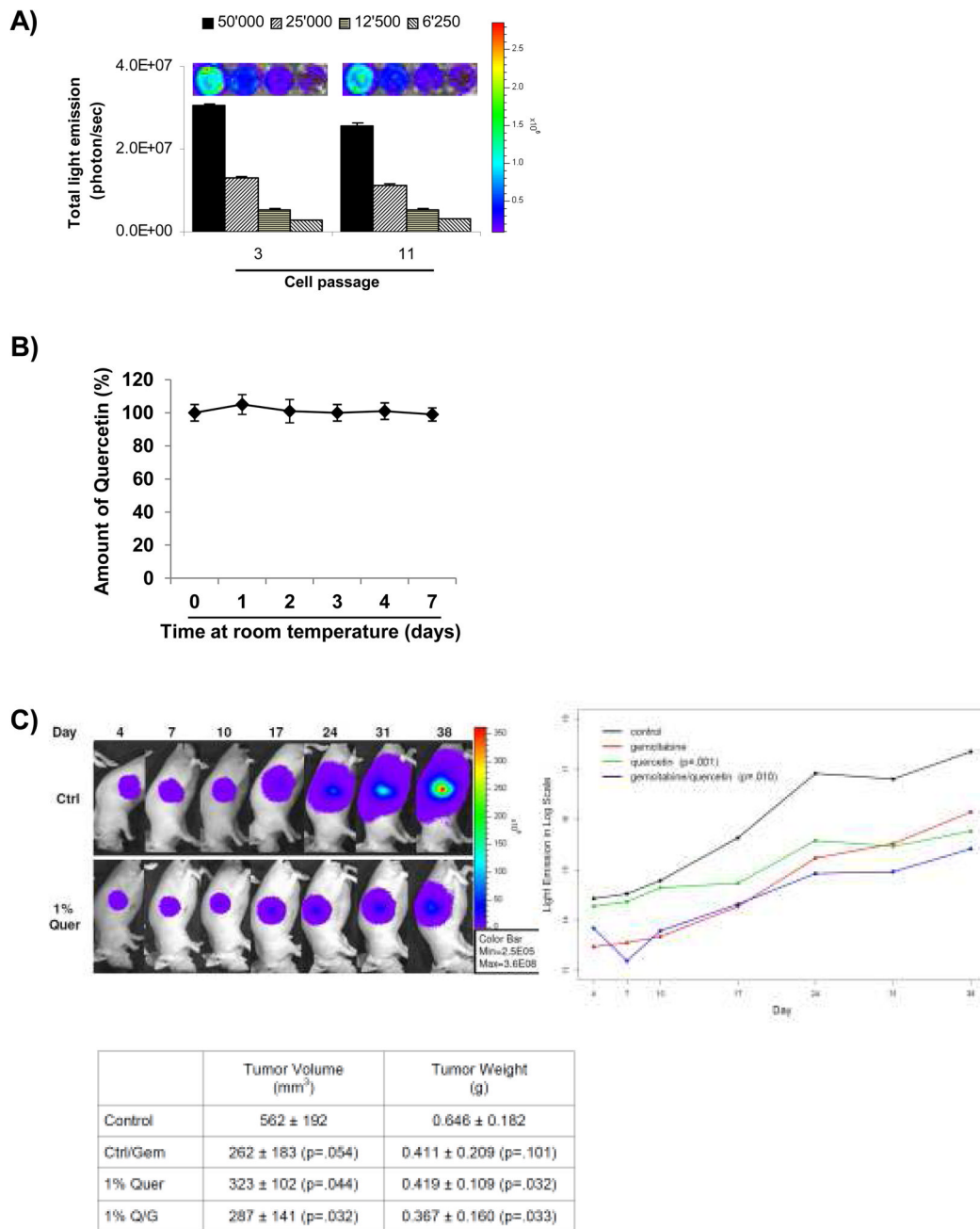
Figure 1.
MIA PaCa-2 and BxPC-3 cells were treated with quercetin (10, 30, 50, and 75 μM), gemcitabine (0.001, 0.01, 0.1, 1, and 10 μg/ml), or vehicle for 48 hours. Cell proliferation was assessed by (A) cell count and (B) MTT. *, p<0.01 vs. quercetin 0 μM or gemcitabine 0 μg/ml. The insets (A) show cells treated with 75 μM quercetin or vehicle with or without 500 U/ml catalase for 48 hours. *, p<0.01 vs. control. C) MIA PaCa-2 and BxPC-3 cells were treated with the control vehicle, gemcitabine (G; 0.001 μg/ml), quercetin (Q; 10 μM), or gemcitabine (G; 0.001 μg/ml) and quercetin (Q; 10 μM) for 48 hours. Cell growth was assessed by cell count. Results were normalized to vehicle treated cells. *, p<0.01 vs. control; **, p<0.05 vs. control; n.s. = not significant
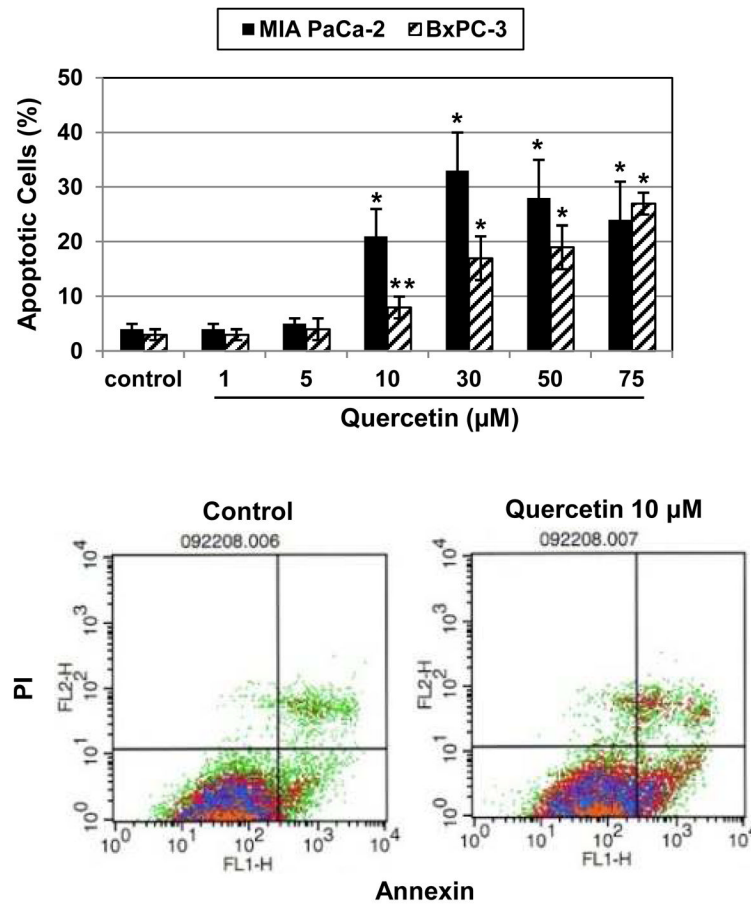
Figure 2.
MIA PaCa-2 and BxPC-3 cells were treated with quercetin (1, 5, 10, 30, 50, and 75 µM) or control vehicle for 48 hours. Cell death was assessed by flow cytometry after Annexin/PI staining. Apoptotic cells are presented as percentage of total counted cells. *, p<0.01 vs. control; **, p<0.05 vs. control. Representative flow cytometry of MIA PaCa-2 cells treated with 10 µM quercetin or control vehicle. Apoptotic cells cluster in the lower right quadrant.

A) 50'000, 25'000, 12'500, 6'250

Total light emission (photon/sec) vs Cell passage (3, 11)

B) Amount of Quercetin (%) vs Time at room temperature (days)

C) Day 4, 7, 10, 17, 24, 31, 38. Ctrl, 1% Quer. Light Emission in Log Scale vs Day.

Legend: control; gemcitabine; quercetin (p=.001); gemcitabine/quercetin (p=.010)

|	Tumor Volume (mm³)	Tumor Weight (g)
Control	562 ± 192	0.646 ± 0.182
Ctrl/Gem	262 ± 183 (p=.054)	0.411 ± 0.209 (p=.101)
1% Quer	323 ± 102 (p=.044)	0.419 ± 0.109 (p=.032)
1% Q/G	287 ± 141 (p=.032)	0.367 ± 0.160 (p=.033)

(Note: In parenthesis is p-value of t-test versus control.)

Figure 3.
A) MIA PaCa-2 cells were transduced with a lentiviruses expressing firefly luciferase. Cells were serially diluted and stable light emission was confirmed by *in vitro* cell culture over 8 passages (passage 3 to 11) by bioluminescence after adding luciferin to the cells. B) The stability of quercetin in the mouse diet was examined by HPLC analysis. Diet was kept at room temperature for 0 to 7 days. Stability of quercetin is presented as the amount of quercetin present at the respective day in percentage of the original amount (day 0). C) Representative real-time bioluminescence imaging over time (days 4–38) of orthotopically grown pancreatic tumors in mice fed the control diet or 1% Quercetin-supplemented diet (left panel). Mean light emission over time (days 4–38) of treatment with control vehicle,

quercetin (1%), gemcitabine (120 mg/kg i.p.), or quercetin (1%) and gemcitabine (120 mg/kg i.p.) (right panel). Tumor volume and tumor weight (mean ± SD; n=6 in each group) at harvest after 42 days of treatment with control vehicle, quercetin (1%), gemcitabine (120 mg/kg i.p.), or quercetin (1%) and gemcitabine (120 mg/kg i.p.)
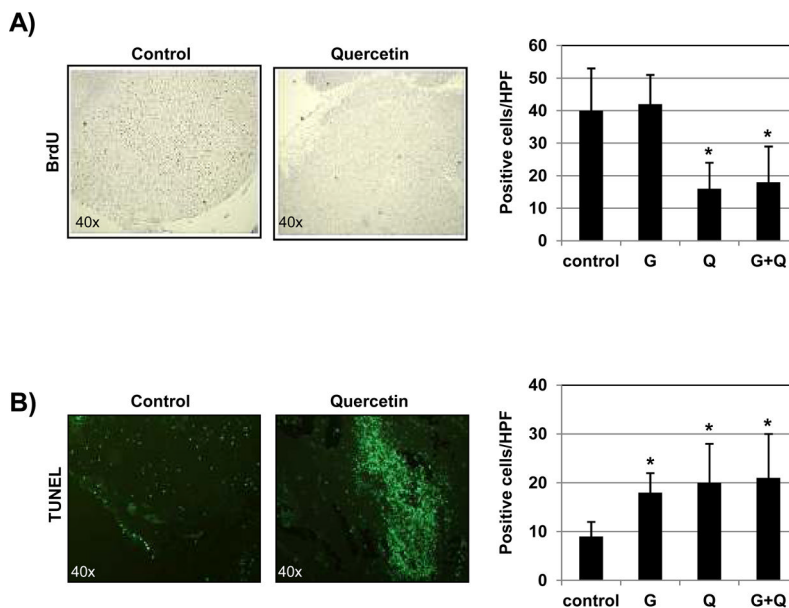
Figure 4.
(A) Representative images of BrdU staining of pancreatic tumors in mice treated with control vehicle (left) or 1% quercetin (right). Original magnification: 40x. The right panel shows the quantitative analysis of BrdU staining as number of positive cells per high power field (HPF) of pancreatic cancers in mice treated with control vehicle, gemcitabine (G; 120 mg/kg i.p.), quercetin (Q; 1%), or gemcitabine (G; 120 mg/kg i.p.) and quercetin (Q; 1%). *, $p < 0.05$ vs. control. (B) Representative images of TUNEL staining in pancreatic tumors in mice treated with control vehicle (left) or 1% quercetin (right). Original magnification: 40x. Right panel shows the quantitative analysis of TUNEL staining as number of positive cells per high power field (HPF) of pancreatic cancers in mice treated with control vehicle, gemcitabine (G; 120 mg/kg i.p.), quercetin (Q; 1%), or gemcitabine (G; 120 mg/kg i.p.) and quercetin (Q; 1%). *, $p < 0.05$ vs. control.