Inhibition of Integrin-Linked Kinase by a Selective Small Molecule Inhibitor, QLT0254, Inhibits the PI3K/PKB/mTOR, Stat3, and FKHR Pathways and Tumor Growth, and Enhances Gemcitabine-Induced Apoptosis in Human Orthotopic Primary Pancreatic Cancer Xenografts

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Abstract

Integrin-linked kinase (ILK) couples integrins and growth factors to downstream signaling pathways involving phosphatidylinositol 3-kinase, protein kinase B/Akt (PKB/Akt), and glycogen synthase kinase-3. The anticancer effects of ILK inhibitor QLT0254 were tested in an orthotopic primary xenograft model of pancreatic cancer. The pharmacodynamic effects of a single dose of QLT0254 on the phosphorylation of PKB/Akt were measured by immunohistochemistry and Western blotting, and showed a decrease of >80% after 2 hours, followed by recovery over 24 hours, consistent with the pharmacokinetic profile of this compound in mice. There was also suppression in phosphorylated PKB Thr308, forkhead in rhabdomyosarcoma, S6K1, S6, 4E-BP1, and signal transducers and activators of transcription 3 Tyr705 and Ser727 protein levels with ILK inhibition by QLT0254. However, we did not observe an effect on phosphoinositide-dependent kinase 1, glycogen synthase kinase-3, and extracellular signal-regulated kinase phosphorylation or on total PKB and ILK protein expression levels with QLT0254 treatment. In tumor growth inhibition experiments, daily treatment with QLT0254 for 3 weeks was well tolerated and produced significant tumor growth inhibition compared with vehicle control (P = 0.001). When a single dose of QLT0254 and chemotherapy agent gemcitabine was administered, there was a significant 5.4-fold increase in acute apoptosis in the combination therapy group compared with vehicle controls (P = 0.002). However, the acute effects of QLT0254 on proliferation were not statistically significant. These results show in vivo evidence that ILK plays a prominent role in oncogenic phosphatidylinositol 3-kinase/PKB signaling in vivo with major impact on the mammalian target of rapamycin, signal transducers and activators of transcription 3, and forkhead in rhabdomyosarcoma signaling pathways, suggesting that ILK inhibitors might show activity in pancreatic cancer patients. (Cancer Res 2005; 65(4): 1497-1504)

Introduction

Pancreatic adenocarcinoma is the fifth leading cause of cancer death in North America, with a mortality rate virtually identical to the incidence rate (1). To date, the only active chemotherapy agent, gemcitabine (2, 2‘-difluorodeoxycytidine), has an objective response rate of <20% (2). Several studies as well as our own have previously shown that perturbation to the frequently up-regulated phosphoinositide 3-kinase-protein kinase B (PI3K-PKB) cell survival pathway in pancreatic cancer confers therapeutic benefits (3–5). Inhibition of PI3K led to decreased proliferation and increased apoptosis in pancreatic cancer in vitro and in vivo (5). In addition, combining PI3K or PKB inhibitors with cytotoxic drugs resulted in the reversal of drug resistance in pancreatic cancer cells and orthotopic xenografts (3–7). Therefore, altering molecular targets pharmacologically in the PI3K-PKB pathway has proven to be a fruitful approach for improving the therapeutic index in pancreatic cancer models.

Recently, the integrin-linked kinase (ILK) has been identified as an important member of the PI3K-PKB pathway. ILK is an ankyrin repeat–containing serine-threonine protein kinase that mediates a diversity of cell functions by coupling integrins and growth factors to cascades of downstream signaling events. This protein is widely expressed throughout the body, with high levels of expression in the pancreas, and the cardiac and skeletal muscles (8, 9). Overexpression of ILK in mammary and intestinal epithelial cells leads to the stimulation of anchorage-independent cell growth, cell cycle progression, and constitutive up-regulation of cyclins D and A expression and tumorigenicity in nude mice (10, 11). Furthermore, elevated ILK expression and activity have been correlated with melanoma (12) as well as with cancers of the breast, prostate, brain, colon, stomach, and ovary (13–18). Hence, ILK seems to be important in carcinogenesis.

ILK is a downstream substrate of PI3K and an important upstream kinase for the regulation of PKB/Akt (9). For PKB to become fully activated, it must be recruited to the plasma membrane and become phosphorylated at both Thr308 and Ser473 sites (19). Thr308 is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) and recent studies have shown that in certain circumstances, ILK is involved in the phosphorylation of Ser473 site. Fully activated PKB can then mediate diverse signaling events through the phosphorylation of glycogen synthase kinase-3β (GSK-3β), forkhead in rhabdomyosarcoma (FKHR) transcription factor, and the mammalian target of rapamycin (mTOR) pathways. GSK-3β has been proposed to be phosphorylated and
inhibited directly by ILK or indirectly through the fully active phosphorylated form of PKB (20). The inhibition of GSK-3β results in the activation of β-catenin/lymphoid enhancing factor 1 and activator protein 1 transcription factors as well as the stabilization and accumulation of cyclin D1 in the nucleus leading to activated transcription, cell cycle progression, and proliferation (10, 20). The phosphorylation of FKHR by PKB, on the other hand, is followed by nuclear exclusion and inhibition in the expression of FOXO-regulated genes such as the cell cycle inhibitor p27kip1 and proapoptotic molecules Fasl and Bim (21, 22). Additionally, the activation of the mTOR pathway by PKB phosphorylates signal transducers and activators of transcription 3 (Stat3) Ser277, resulting in transcription of genes involved in cell differentiation, proliferation, and apoptosis (23); 4E-BP1, thereby releasing transcription factor eIF-4E for DNA binding and ultimately increasing protein synthesis (24); and S6K1 and its downstream substrate S6 ribosomal protein, leading to increased cell growth (24, 25). Recently, it has been reported that both S6K1 and 4E-BP1/eIF-4E pathways are required for and independently mediate mTOR-dependent G1 phase progression (26). Therefore, the activation of PKB by ILK has the potential for extensive downstream effects in the regulation of cell signaling pathways. Consequently, deregulation of the PI3K/ILK/PKB pathway might play a major role in cancer development and growth.

Materials and Methods

Primary Pancreatic Cancer Xenograft and Orthotopic Model. Animal experiments were done using protocols approved by the University Health Network Animal Welfare Committee. The establishment of the primary pancreatic cancer xenografts was previously described (27). Ontario Cancer Institute Pancreas #4 (OCIP#4) is a primary pancreatic cancer xenograft continuously propagated in severe combined immunodeficiency (SCID) mice for in vivo drug testing. Each 6-week-old male SCID mouse was anesthetized and an incision was made in the upper left abdomen exposing the pancreas. Tumor pieces from the previous passage were attached to the pancreas, which was then returned to the peritoneum. Drug treatment commenced after 4 weeks of tumor growth.

Drug Preparation. ILK inhibitor QLT0254 was obtained from Kinetek Pharmaceuticals, Inc. (recently acquired by QLT Inc., Vancouver, British Columbia, Canada). QLT0254 has been shown to inhibit the kinase activity of ILK in cell-free assay at 185 nmol/L and preliminary experiments suggest that it has ~ 100-fold selectivity over other kinases tested under similar conditions, including CDK2, CDK5, CK2, CSK, ERK1, GSK3α, LCK, PIM1, PKA, DNA-PK, and PKB/Akt (QLT, Inc.). Of those tested, CDK1, PKC, and FYN show the greatest inhibition by QLT0254 but the selectivity window is 100-fold over other kinases tested under similar conditions.

Treatment Protocols. In the time course experiment, five groups of three randomly assigned, tumor-bearing OCIP#4 SCID mice were treated with 200 mg/kg QLT0254 i.p. (except for the 0 h group) before sacrificing and harvesting the tumors at 0, 2, 4, 8, and 24 hours.

In the tumor growth inhibition experiment, the QLT0254 dose was reduced from 200 to 100 mg/kg. Two groups of eight randomly assigned, tumor-bearing OCIP#4 SCID mice were given QLT0254 (100 mg/kg) or the vehicle control [5% Tween 80 in double-distilled water (ddH2O)]. All treatments were given by i.p. injections, five times consecutively in a week, for a total of 3 weeks. At the end of the third week, the animals were sacrificed and the tumors were harvested and weighed after removal of nontumoral tissues.

Next, the single-dose acute combination therapy experiment was replicated once to include a total of 24 tumor-bearing animals with 6 animals randomly assigned to one of four groups: (a) drug-vehicle controls (PBS and 5% Tween 80 in ddH2O, i.p.); (b) gemcitabine (80 mg/kg, i.p.); (c) QLT0254 (150 mg/kg, i.p.); and (d) gemcitabine and QLT0254 (80 and 150 mg/kg, respectively, i.p.). For group d, gemcitabine was given at 0 hour followed by QLT0254 at 24 hours. A similar schedule was used in the other groups except that the drug not included in the treatment regimen was replaced with the corresponding vehicle. All mice were killed 48 hours after beginning the experiment.

Last, the 4-week combination therapy experiment consisted of the same sample size and treatment groups as the previous single-dose acute combination therapy experiment; however, in this experiment both gemcitabine and QLT0254 were administered i.p. twice a week, with gemcitabine on Mondays and Thursdays and the ILK inhibitor on Tuesdays and Fridays for the combination group. Again, other groups followed a similar schedule with the exception that the drug not included in the regimen was substituted with the corresponding vehicle. After 4 weeks of treatment, the animals were killed and the tumors were harvested and weighed immediately.

Depending on the subsequent analyses, all harvested tumors were either cut into pieces and fixed in 10% formalin for immunohistochemistry, snap-frozen in OCT (Miles, Inc., Elkhart, IN) in liquid nitrogen for immunofluorescence or treated with lysis buffer for Western blots.

Western Blot Analysis. Minced tumor pieces were homogenized in 1 ml lysis buffer [50 mmol/L HEPES (pH 8.0), 10% glycerol, 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 1.5 mmol/L MgCl2, 100 mmol/L NaF, 10 mmol/L Na3PO4, 0.1 mmol/L NaVO4] containing protease inhibitor cocktail tablets (Roche Canada, Mississauga, Ontario, Canada) for 1 hour on ice. Homogenates were clarified by centrifuging at 14,000 rpm at 4°C for 15 minutes. Samples were then heated in sample buffer for 10 minutes at 95°C, run on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes using the Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Membrane blots were blocked for 1 hour at room temperature with 10% nonfat milk in TBS with 0.1% Tween 20 and then incubated overnight at 4°C with the following primary antibodies: PKB (1:500, Cell Signaling Technology, Beverly MA), ILK1 (1:500), P-PKB/Akt Ser473 (1:1000), P-PKB Thr389 (1:500), P-PDK1 Ser241 (1:500), P-GSK3β Ser9 (1:1000), P-p70S6K Thr389 (1:1000), P-Stat3 Ser727 (1:1000), P-Stat3 Ser705 (1:500), and β-actin (1:25,000, Abcam, Cambridge, MA). The blots were then probed with either the anti-rabbit polyclonal or anti-mouse monoclonal secondary antibody containing horseradish peroxidase (Amersham Biosciences, Baie d’Urfe, Quebec, Canada) at 1:1,000 the next morning for 1 hour at room temperature before exposing to ECL (Amersham Biosciences) per manufacturer’s instructions. Blots were either incubated once with a primary antibody or stripped once, blocked, and reprobed with a different antibody overnight at 4°C. Densitometric analyses for P-PKB/Akt Ser473 were done using densitometry and ImageQuant v.3.0 (Molecular Dynamics, Sunnyvale, CA).

Immunohistochemistry. Paraffin-embedded sections of OCIP#4 tumor tissues from the time course experiment were stained with H&E, monoclonal ILK antibody (1:200, Upstate Biotechnology, Lake Placid, NY), phosphospecific polyclonal antibodies PKB/Akt Ser473 (1:400) and GSK-3β Ser9 (1:500), and developed with a streptavidin/biotin-peroxidase method.

Indirect Immunofluorescence. Indirect immunofluorescence was used for the frozen sections obtained from the tumor growth inhibition and the acute combination therapy experiments. Serial sections (5-µm-thick) were cut from OCT-embedded frozen tissue and fixed in 2% formaldehyde for

5 A. Stewart, personal communication.

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20 minutes. One section was stained with H&E for the selection of the tumoral areas with transmitted light microscopy. The other sections were stained with the following: (a) CD31 (1:500, BD Biosciences, Franklin Lakes, NJ) and labeled with the Cy3-conjugated rat anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA) for detection of vasculature, (b) proliferation marker Ki-67 and the early apoptosis marker, cleaved caspase-3 (1:100, DAKO, Glostrup, Denmark; 1:50, Cell Signaling Technology) with secondary antibodies Alexa 488 and a Cy5-conjugated anti-mouse secondary antibody (Jackson Immunoresearch) respectively. Controls for nonspecific background were used by staining sections with secondary antibody alone. All sections were counterstained with DNA specific dye 4',6-diamidino-2-phenylindole to outline the nuclear area.

**Computerized Image Analysis.** Composite digital images of the entire H&E sections were acquired as described previously (28). On these H&E images, the total viable tumor area was selected for analysis of the double-labeled Ki-67/cleaved caspase-3 sections, whereas the viable tumor and stroma of the viable tumor were selected for analysis of the CD31 marker.

The selected tumoral areas of the CD31 and the Ki-67/cleaved caspase-3 sections were examined using a Microcomputer Imaging Device image analysis system (Imaging Research Inc., St. Catharines, Ontario, Canada) equipped with a cooled charged couple device camera mounted on an epifluorescence microscope. For each tumor, the analyzed area was obtained by tiling an entire section at 20× magnification (2.24 mm²) to create composite images of individual fields as previously described (29).

To examine Ki-67 staining (nuclear), the nuclear image obtained with 4',6-diamidino-2-phenylindole was binarized to form masks, which were then overlaid onto the Ki-67 fluorescence image to capture Ki-67 staining only within the nucleus. A size criterion (mean value of positive Ki-67 nuclei) was selected to count the number of positive objects. The percentage of nuclear Ki-67 was then acquired by normalizing the number of positive nuclear Ki-67 objects over the number of nuclei stained with 4',6-diamidino-2-phenylindole and multiplying by 100. Cleaved caspase-3 was measured as the percentage of positively stained area that is the proportional area (total cleaved caspase-3 positive area/total viable tumoral area) expressed as a percentage. The CD31 images were also binarized and measured as the number of positive objects per tumoral area for total vascular area (all vessels > 20 μm²) and average proportional area (total viable tumoral area for total vascular area > 20 μm²).

**Statistics.** All values are presented as mean ± SE. Comparisons between the two groups (control versus QLT0254) were achieved using the two-tailed Student’s t test, whereas comparisons between four treatment groups in the single-dose acute combination therapy experiment and the 4-week combination therapy experiment were done using Kruskal-Wallis one-way ANOVA on ranks and subsequently the Student-Newman-Keuls method for all pairwise multiple comparisons between the groups. The criterion for statistical significance is \( P < 0.05 \).

**Results**

**Molecular Effects of a Single Dose of QLT0254 in Orthotopic Primary Pancreatic Xenografts.** The QLT0254 dose of 200 mg/kg administered i.p. to the time course experimental animals was close to the maximum tolerated dose. The molecular targets examined are shown in Fig. 1. First, we assessed the effects of QLT0254 on P-PKB/ Akt Ser\(^{473}\) protein levels using immunohistochemistry and Western blots. Figure 2 displays the immunohistochemical staining of ILK in the untreated OCIP#4 orthotopic tumors. OCIP#4 shows typical features of pancreatic ductal carcinoma with cancer cells loosely arranged in glandular structures embedded in an extensive fibrovascular stroma. Immunoreactivity to ILK was stronger in the tumor stroma and some endothelial cells than in tumor cells. For the time course experiment, strong P-PKB/Akt Ser\(^{473}\) staining was evident in the tumor and the tumor stroma of untreated OCIP#4 at 0 hour (Fig. 2A). Significant suppression of P-PKB/Akt Ser\(^{473}\) was found after 2 hours of QLT0254 administration (Fig. 2C) followed by gradual recovery to pretreatment levels at 24 hours (Fig. 2D,F). Consistent with the immunohistochemical images, Western blots as well as its densitometry analysis (Fig. 3A and B, respectively) also showed significant inhibition of P-PKB/Akt Ser\(^{473}\) levels by 7-fold after 2 hours, 5-fold after 4 hours, and 4.5-fold after 8 hours.

![Figure 1](image1.jpg)

**Figure 1.** PI3K/ILK/PKB pathway with cross-talk from the MAPK pathway and related proteins.

![Figure 2](image2.jpg)

**Figure 2.** Immunohistochemical staining of (A) ILK in untreated OCIP#4 orthotopic pancreatic tumors and (B-F) phosphorylated PKB/Akt Ser\(^{473}\) in OCIP#4 orthotopic pancreatic tumors after a single dose of 200 mg/kg QLT0254 at indicated time points.
At 24 hours, PKB/Akt Ser↑73 phosphorylation recovered to pretreatment levels. Contrary to findings from in vitro studies, where the ILK inhibitors did not decrease phosphorylated PKB at Thr↑308 (30), we observed a slight decrease in phosphorylated PKB/Akt Thr↑308 protein levels from 2 to 8 hours after QLT0254 treatment (Fig. 3A). This prompted us to examine the protein levels of the active phosphorylated form of PKD1 because P-PDK1 is responsible for the phosphorylation of PKB at Thr↑308 (31, 32). However, phosphorylated PKD1 was unaffected by QLT0254 treatment (Fig. 3A). In addition, PKB and ILK protein levels were unchanged after drug treatment (Fig. 3A).

Figure 3C shows the effects of a single dose of 200 mg/kg QLT0254 on the putative ILK downstream targets GSK-3β, ERK, and FKHR. Both ILK and PKB can phosphorylate and inhibit GSK-3β at Ser↑9 (20). Hence, we examined the protein levels of phosphorylated GSK-3β at Ser↑9 in Western blots (Fig. 3C) and immunohistochemistry (not shown) and observed no effect within 24 hours of QLT0254 administration in OCIP#4 tumors. Moreover, ERK, which has been shown to be activated by ILK to regulate muscle differentiation (33), remained phosphorylated despite ILK inhibition (Fig. 3C). Ser↑256 phosphorylation of the PKB substrate, FKHR, was substantially decreased after 2 hours of QLT0254 treatment and this effect persisted throughout the time course of 24 hours.

Figure 3D shows the Western blots of the two well-characterized substrates downstream of the mTOR pathway. The two isoforms p70S6K and p85S6K, together termed S6K1, were detected in these OCIP#4 lysates in the phosphorylated form, with the nuclear p85S6K protein having much higher basal levels at 0 hour after very short exposure to film than the cytoplasmic p70S6K isoform, which appeared as a faint band after longer exposure. Treatment with QLT0254 decreased phosphorylated p85S6K at the mTOR-regulated Thr↑412 site after 2 hours of treatment and this effect remained until slight recovery at 24 hours. The P-p70S6K isoform also showed dephosphorylation at 2 hours, but this effect was not as pronounced.

S6 ribosomal protein is the primary substrate phosphorylated by P-S6K1 (Fig. 1). As shown in Fig. 3D, P-S6 was considerably dephosphorylated at residues Ser↑240/244, with slight recovery from 4 to 24 hours. Conversely, Ser↑235/236, another residue of S6, also becomes dephosphorylated but to a lesser extent than the Ser↑240/244 site. At Ser↑235/236, dephosphorylation occurs slightly after 2 hours but phosphorylation returned after 8 hours. Recently, it was shown that in the absence of S6K1 and S6K2 (an isoform of S6K1) activity, a MAPK pathway cooperates in the regulation of S6 phosphorylation at Ser↑235/236 (34). Therefore, the weaker dephosphorylation pattern of S6 at Ser↑235/236 than Ser↑240/244 may be due to signaling from the MAPK pathway in the absence of S6K1 and perhaps S6K2, but we did not examine the protein levels of S6K2 in this study. This conforms to the finding that P-ERK remained unaffected with treatment (Fig. 3C). Another important substrate, 4E-BP1, undergoes inhibitory phosphorylation by mTOR. Inhibition of ILK with QLT0254 led to an almost complete ablation of this protein after 2 hours and recovery at 4 hours.

Stat3, another target of mTOR, is phosphorylated at Ser↑727 (Fig. 1) and QLT0254 treatment led to a dephosphorylation after 2 hours with recovery after 8 hours (Fig. 3E). The tyrosine site of Stat3, on the other hand, is phosphorylated by cytokine receptors through JAK or directly through activated receptor and nonreceptor
tyrosine kinases at the plasma membrane before its dimerization and translocation to the nucleus for phosphorylation of the serine site by mTOR (23) or by ERK (35). This tyrosine site, however, becomes dephosphorylated slightly by 4 to 8 hours, with more dephosphorylation at 8 hours before reappearance of the activated form at 24 hours (Fig. 3E). PKB protein levels show equal loading of samples. Similar results were obtained from tumors of three different animals killed at each time point. Overall, these results show that a single i.p. dose of QLT0254 at 200 mg/kg is able to dephosphorylate PKB, FKHR, S6K1, S6 ribosomal protein, 4E-BP1, and Stat3 without affecting the protein levels of PKB, ILK, P-PDK1, P-GSK-3β, and P-ERK in the OCIP#4 human orthotopic pancreatic tumors (Fig. 1).

**Tumor Growth Inhibition after 3-Week QLT0254 Treatment as a Single Agent.** There were three deaths in the control group before the end of the 3-week treatment because of large tumor. QLT0254 was well tolerated in the tumor-bearing SCID mice. All animals in this group were alive and exhibited a healthier appearance than the control animals. On average, the control mice lost 1.31 g from the initial weight of 24.66 g, whereas QLT0254-treated mice lost only 0.47 g from the initial 25.47 g but the difference in weights between the two groups at the termination of the study were not statistically significant. Because of the location of the tumor in the orthotopic pancreas site, the tumor size could not be measured until at the end of the experiment when the animals were sacrificed. Consequently, at the end of the 3-week treatment, orthotopic tumors were dissected free of surrounding normal tissues and weighed. As shown in Fig. 4A, treatment with 100 mg/kg of QLT0254 five times a week for a total of 3 weeks significantly inhibited tumor growth by 2-fold compared with vehicle-treated controls (P = 0.004).

**Angiogenesis Effects of 3 Weeks QLT0254 Administration in Orthotopic Pancreatic Tumors.** Indirect immunofluorescence of CD31-Cy3 images showed OCIP#4 as relatively vascularized tumors. Computerized image analysis of these images from control and treated groups found no effect on tumor angiogenesis as determined by the total vascular area (Fig. 4B) and the number of microvessels per tumoral area (microvascular density) in Fig. 4C (total vascular area, P = 0.287; number of microvessels/tumoral area, P = 0.393).

**Effects of 3 Weeks Administration of QLT0254 on Proliferation and Apoptosis.** Indirect immunofluorescence staining for nuclear Ki-67 in the tumor growth inhibition tumors was used. Figure 4D displays a bar graph of the percentage of nuclear Ki-67 in the vehicle control and QLT0254-treated tumors. Although on average, QLT0254-treated tumors revealed less Ki-67 staining than the control tumors, the difference between the means was statistically insignificant (P = 0.323).

Figure 4E compares the percentage of positively stained cleaved caspase-3 area between the control and QLT0254-treated tumors. The data showed no statistically significant effect of QLT0254 in inducing apoptosis in QLT0254-treated tumors compared with vehicle controls (P = 0.369). The same sections were also stained for terminal deoxynucleotidyl transferase–mediated nick end labeling and revealed similar results (data not shown). Nonetheless, there was a trend toward more apoptosis and less proliferation in

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**Figure 4.** Effects of 3 weeks QLT0254 treatment on tumor weight, angiogenesis, proliferation, and apoptosis of OCIP#4 orthotopic primary pancreatic xenografts. A, tumor weight in grams of OCIP#4 orthotopic xenografts treated with vehicle control (5% Tween 80 in ddH2O, i.p.; n = 8) or QLT0254 (100 mg/kg, i.p.; n = 8) five times a week for 3 weeks. Statistically significant difference was observed between QLT0254-treated tumors and vehicle controls (P = 0.004). Comparison of the total vascular area of CD31 (B) and the number of positive CD31 objects per tumoral area (C) between the controls and QLT0254-treated tumors. There was no statistically significant difference between the control and QLT0254-treated groups (n = 8; B, P = 0.287; C, P = 0.393). D, percentage of nuclear Ki-67 was compared between the control and QLT0254-treated tumors but the difference was not statistically significant (P = 0.323). The percentage of positive cleaved caspase-3 area (E) was compared between the control and QLT0254-treated group and no statistically significant difference was observed (P = 0.369). Bars, SE.
QLT0254-treated tumors and more proliferation and less apoptosis in the control tumors.

**Single-dose Acute Proliferative and Apoptotic Effects of Combination Therapy.** Figure 5A compares the percentage of nuclear Ki-67 between the drug-vehicle control group and the xenografts treated with a single dose of 150 mg/kg QLT0254 i.p. for 24 hours. In general, there was a decrease in Ki-67 labeling in QLT0254-treated mice but this effect was not statistically significant (P = 0.374).

The percentage of positive cleaved caspase-3 area between the four groups, drug-vehicle controls (5% Tween 80 in ddH2O), gemcitabine (80 mg/kg for 48 h), QLT0254 (150 mg/kg for 24 h), and combined gemcitabine and QLT0254 (80 mg/kg for 48 hours and 150 mg/kg for 24 hours) was measured, as shown in Fig. 5B. There was a statistically significant increase in apoptosis induced by either agent given alone and in the combined treatment of gemcitabine and QLT0254 compared with vehicle controls (P = 0.002). In addition, induction of apoptosis by either agent given alone was statistically different than the combined therapy where apoptosis was enhanced (P = 0.002). However, there was no statistically significant difference in apoptotic effects between gemcitabine and QLT0254 when treated as single agent (P > 0.05).

**Tumor Growth Inhibition after 4-Week Combination Therapy.** Figure 6 compares the tumor weights of OCIP#4 animals treated with i.p. injections of the vehicle control, 80 mg/kg of gemcitabine twice a week, 100 mg/kg of QLT0254 twice a week, or in combination with 80 mg/kg of gemcitabine on Mondays and Thursdays and 100 mg/kg of QLT0254 on Tuesdays and Fridays for 4 weeks. There were significant differences between the control versus the gemcitabine alone group, control versus combined treatment group, gemcitabine alone versus QLT0254 alone group, and QLT0254 alone versus combined treatment group (P < 0.001). Nevertheless, because these tumors were relatively sensitive to gemcitabine alone, there was no statistically significant enhancement in tumor reduction when treatment was combined with QLT0254 (P > 0.05), although there is a suggestion that the addition of QLT0254 to gemcitabine resulted in a further reduction in tumor size. Furthermore, the actual mean tumor weight of the combined treated group is less than that shown in Fig. 6 because histologic examination of the H&E sections in this group revealed microscopic tumors surrounded by substantial amounts of non-tumoral tissue that could not be distinguished from the tumor during the naked eye dissection.

**Discussion**

Our results show that treatment with QLT0254 in vivo can suppress phosphorylated protein levels of PKB/Akt, FKHR, S6K1, S6 ribosomal protein, 4E-BP1, and Stat3 without down-regulating phosphorylated GSK-3β and ERK protein expression or affecting total ILK and PKB protein levels, consistent with selective ILK inhibition. When the drug was administered for 3 weeks, tumor growth was inhibited in the orthotopic primary pancreatic cancer xenografts with a trend toward an increase in apoptosis and decrease in proliferation in the drug-treated tumors compared with controls, although these effects were not statistically significant. Interestingly, QLT0254 also additively increased apoptosis when treated in combination with gemcitabine. Given the high intrinsic resistance of pancreatic cancer, these results provide encouraging support for the targeting of ILK for therapeutic purposes.

The small molecule inhibitor QLT0254 acts to inhibit the catalytic activity of ILK, which in turn can block the phosphorylation of GSK-3β and PKB/Akt Ser473, leading to the incomplete activation of PKB/Akt. Structurally related ILK inhibitors have been tested in phosphatase and tensin homologue–mutated prostate cancer cell lines PC-3 and LNCaP, resulting in the suppression of phosphorylated PKB/Akt at Ser473 but not Thr308 (30). In the present study, we found >80% inhibition of P-PKB/Akt Ser473 levels following a single dose of QLT0254 in vivo, with recovery over 24 hours consistent with the pharmacokinetic profile of the compound. However, we also observed slight dephosphorylation in PKB Thr308 protein, possibly due to minor nonspecific effects of the compound at the relatively high dose of 200 mg/kg (close to maximum tolerated dose) because P-PDK1 was not affected with treatment. Regardless, the inhibitory effects were much more profound at the Ser473 residue. Altogether, these results showed the efficacy of QLT0254 in suppressing ILK and further support the notion that ILK can lead to the phosphorylation of PKB/Akt Ser473.
Tan et al. (36) have shown that other analogues of QLT0254, KP-SD-1, and KP-SD-2 can suppress LS-180 human colon cancer cell and s.c. xenograft tumor growth. In the colon cancer xenografts, the ILK inhibitors blocked PKB/Akt Ser473 and GSK-3β phosphorylation (36). Administration of QLT0254 for 3 weeks to our primary orthotopic pancreatic cancer xenografts also resulted in tumor growth inhibition and suppression of PKB/Akt phosphorylation but with no effects in phosphorylated GSK-3β. The failure to suppress phosphorylation of GSK-3β despite the inhibition of its upstream kinases ILK and PKB by QLT0254 might be attributed to signaling from GSK-3β by other upstream regulators, such as Dishevelled, GBP/FRAT1, PKCo, and PP2A from the Wnt signaling pathway (37), as well as PKA (38, 39) and SGK (40). This sustained phosphorylation of GSK-3β and the consequent proliferation may be one factor leading to the insignificant decrease in Ki-67 labeling in QLT0254-treated tumors compared with controls in both single dose as well as the 3-week tumor inhibition studies.

In addition to down-regulation of P-PKB/Akt Ser473, continuous treatment with QLT0254 for 3 weeks resulted in significant tumor growth delay. The suppression of P-PKB/Akt Ser473 in these tumors might potentially result in the initiation of apoptosis and cell cycle arrest via the dephosphorylation of FKHR and Stat3 (21, 23) or via effects on protein synthesis, cell growth, and proliferation downstream of mTOR through inactivation of S6K1 and activation of 4E-BP1 (24–26). Tan et al. (41) have recently reported that QLT0254 can also inhibit tumor angiogenesis in PC3 prostate cancer xenografts, but we did not see this effect in the OCIP#4 model. Although the proliferation and apoptosis effects of QLT0254 on OCIP#4 were not statistically significant, there was a tendency both toward more apoptosis and less proliferation in QLT0254-treated tumors than the controls, suggesting that these combined effects might be responsible for the significant tumor growth inhibition.

We cannot exclude the possibility that in addition to effects on the malignant cell population in OCIP#4 tumors, growth inhibition by QLT0254 is also mediated by effects on the tumor stroma because this expresses higher levels of ILK. Although the suppression of P-PKB Ser473 in the cancer cells suggests that these are in fact the more important drug target, further work is needed to investigate the role of ILK in tumor/stroma interactions in vivo.

It has been proposed that the activation of the PI3K/PKB pathway can produce drug resistance in pancreatic cancers, although other signaling mechanisms might be involved in some cases (42). Our group and others have shown that PI3K inhibitors are able to sensitize pancreatic cancer cells to gemcitabine and that combined treatment is more effective in xenograft models (3, 4, 7). In accordance with these findings, we found that a single dose of QLT0254 enhanced gemcitabine-induced apoptosis in OCIP#4 xenografts. There was also a suggestion that combined treatment twice weekly was more effective than single agents. However, these data are difficult to interpret because of the relatively high sensitivity of OCIP#4 to gemcitabine. Moreover, potential for ILK inhibition to enhance gemcitabine effects in pancreatic cancers requires further testing using different xenograft models.

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