Integrin-linked kinase is a potential therapeutic target for anaplastic thyroid cancer

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Abstract
We investigated integrin-linked kinase (ILK), a focal adhesion serine-threonine protein kinase, as a new molecular target for treating anaplastic thyroid cancer. ILK mediates cell growth and survival signals and is overexpressed in a number of cancers. Therefore, we hypothesized that inhibition of ILK leads to growth arrest and apoptosis of thyroid cancer cells. According to Western blotting, the level of ILK protein was highly expressed in one papillary (NPA187) and four of five (Hth74, DRO, ARO, KAT4, and K4) anaplastic thyroid cancer cell lines. Immunohistochemical analysis of a human tissue microarray revealed that ILK was highly expressed in anaplastic thyroid cancer but not in normal human thyroid tissue. Treating thyroid cancer cell lines with a new ILK inhibitor, QLT0267, inhibited epidermal growth factor–induced phosphorylation of AKT, inhibited cell growth, and induced apoptosis in the NPA187, DRO, and K4 cell lines. QLT0267 also inhibited the kinase activity of immunoprecipitated ILK in four of five cell lines. Tumor volumes in mice treated with QLT0267 were significantly reduced compared with those in untreated mice. In immunohistochemical studies, QLT0267 suppressed phosphorylated p-AKT and angiogenesis (i.e., reduced mean vascular density) and induced apoptosis in both tumor cells and tumor-associated endothelial cells of the thyroid DRO xenografts. In summary, we found that ILK expression and activity were elevated in human anaplastic thyroid cancer and ILK inhibition led to growth arrest and apoptosis in vitro and in vivo. Our results provide preliminary evidence that ILK is a potential therapeutic target for treating anaplastic thyroid cancer. [Mol Cancer Ther 2005;4(8):1146–56]

Introduction
In the United States, thyroid cancer accounts for about 1% of all new cases of cancer. Pathologically, 94% of thyroid cancers are differentiated thyroid carcinomas (i.e., papillary and follicular thyroid cancer; ref. 1). Another 5% are medullary tumors of a neuroendocrine origin. The remaining 1% of thyroid cancers is anaplastic (2). Although the incidence of anaplastic thyroid cancer is low, this disease has a major effect because the associated mortality rate is nearly 100% (3–8). In a study of 15,700 patients with thyroid cancer in the United States, the overall 10-year relative survival rate, corrected for age and sex, was 13% for anaplastic disease (9). The median survival time after diagnosis is 3 to 7 months, and a worse prognosis is associated with large tumors, distant metastasis, acute obstructive symptoms, and leukocytosis (10, 11). Despite the widespread use of multimodality treatment, survival rates have not improved much in the past few decades (12, 13). Patients presenting with widespread local invasion often develop distant metastasis in the lungs, pleurae, bone, and brain (1). Treatment of anaplastic cancer is generally palliative only; thus, new targets for molecular therapy of this cancer are needed.

Among the molecular targets that show promise in the fight against anaplastic cancer is integrin-linked kinase (ILK), a recently identified protein serine/threonine kinase that was discovered through its interactions with the β1 and β3 integrin subunits (14). In general, integrin-mediated interactions of cells with components of the extracellular matrix regulate cell survival, proliferation, differentiation, and migration (15). ILK has four noncatalytic ankyrin repeats that aid in targeting it to focal adhesion complexes and linking it to growth factor receptor tyrosine kinase signaling (16–18).

ILK has a number of oncogenic properties. First, in epithelial cells, forced expression of wild-type ILK suppresses suspension-mediated apoptosis (i.e., anoikis) and stimulates anchorage-independent growth (19). Second, ILK overexpression tends to constitutively activate integrin-signaling pathways so that the anchorage state is mimicked in the absence of cell-extracellular matrix interactions (20). Third, ILK is involved in enhancing tumor cell invasion (21) and tumorigenicity in nude mice (22). Fourth, ILK is overexpressed in colon (23) and prostate tumors (24). Moreover, ILK expression is closely

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correlated with the invasion and metastasis of gastric cancer (25). In addition, ILK phosphorylates a major protein called protein kinase B or AKT kinase on Ser^473 and activates it in PTEN mutant prostate cancer cells (26) and directly phosphorylates GSK-3β, thereby inhibiting its activity (27). Recently, it has been shown that conditional knockout of ILK results in almost complete inhibition of phosphorylation on Ser^473, significant inhibition of protein kinase B or AKT kinase activity, and suppression of phosphorylation of GSK-3β on Ser^7 and cyclin inhibition of D1 expression (28, 29). Moreover, ILK plays a key role in mediating tumor angiogenesis (30).

Until now, the role of ILK expression and activity in thyroid carcinomas has not been studied; the use of ILK inhibitors in cancer therapy is in its early stages. Identifying the role of ILK in thyroid cancer and the use of new ILK inhibitors in thyroid cancer treatment has the potential to identify a new molecular and therapeutic target that is urgently needed in the face of the poor survival rates for patients with anaplastic thyroid cancer.

In this study, we hypothesized that ILK is overexpressed in anaplastic and papillary thyroid cancers and that its inhibition leads to cell cycle arrest and apoptosis. We investigated the expression of ILK in a panel of thyroid cancer cell lines, xenografts, and human thyroid cancer specimens. We also studied the effects of a novel ILK inhibitor on ILK kinase activity, cell growth, and survival of thyroid cancer cells in vitro and on anaplastic thyroid cancer xenografts growth in vivo.

Materials and Methods

Cell Lines and Culture Conditions

The anaplastic thyroid carcinoma cell lines KAT-4, K18, Hth74, ARO, and DRO were used. NPA187 thyroid cancer cell line represented the less aggressive yet the most prevalent form of thyroid cancer. PC3P, a prostate cancer cell line, was used as a positive control for ILK expression. The cells were grown in DMEM supplemented with 10% fetal bovine serum, L-glutamine, penicillin, sodium pyruvate, nonessential amino acids, and a 2-fold vitamin solution (DMEM 10% fetal bovine serum; Life Technologies, Inc., Grand Island, NY). Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% carbon dioxide and 95% air. The cultures were found to be free of Mycoplasma species. The cultures were maintained no longer than 12 weeks after recovery from frozen stocks.

Reagents

The ILK inhibitor QLT0267 was obtained from QLT, Inc. (Vancouver, British Columbia, Canada). QLT0267 was developed through the optimization of a lead compound identified in the high-throughput screening of a rationally designed small-molecule library against the target ILK. The initial lead compound was found to be a submicromolar inhibitor of the phosphotransferase activity of ILK towards a specific peptide substrate. An intensive medicinal chemistry effort around this lead compound has identified many structure/activity relationships and allowed for the identification of a bioactive core of the molecule. Further optimization of this molecular core to enhance both in vitro enzyme and cell-based potencies and to improve the pharmacokinetic and pharmacodynamic properties of this class of compounds has lead to the second-generation compound QLT0267. QLT0267 has been shown to inhibit the kinase activity of ILK in cell-free assay at 26 nmol/L and preliminary experiments suggest that it has ~1,000-fold selectivity over other kinases tested under similar conditions, including CK2, CSK, DNA-PK, PIM1, protein kinase B or AKT kinase, and PKC. ~100-fold selectivity over extracellular signal-regulated kinase 1, GSK3β, LCK, PKA, p70S6K, and RSK1 (QLT).6 Of those tested, CDK1, CDK2, and CDK5 show the greatest inhibition by QLT0267 but the selectivity window is still at least 10-fold. The drug was prepared as 10 mg/mL solution by adding PTE vehicle [66.6% polyethylene glycol 300/8.2% Tween 80/25% ethanol (95%)/0.2% citric acid (w/w)] to the powder, sonicating for 10 minutes, and vortexing until fully dissolved. The dosing solutions were prepared in one batch and stored at ~80°C, to be thawed on the day of dosing. For in vitro administration, QLT0267 was dissolved in DMEM to a concentration of 20 mmol/L. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich Corp. (St. Louis, MO). The AKT kinase kit was purchased from Cell Signaling Technology (Beverly, MA).

Materials

ILK antibodies were purchased from Upstate Biosciences (Waltham, MA). For Western blotting, an anti-ILK antibody was used at a dilution of 1:5,000. For immunoprecipitation, an ILK antibody was used. The anti-p-AKT Ser^473 antibody was purchased from Cell Signaling Technology and was used at a dilution of 1:2,000. For secondary antibodies, we used goat anti-mouse secondary antibody from Bio-Rad Laboratories (Hercules, CA) at a dilution of 1:3,000 and a goat anti-rabbit antibody from Santa Cruz Biotechnology (Santa Cruz, CA) at a dilution of 1:3,000. Protein A beads and myelin basic protein were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Measurement of Cytotoxicity

Five thousand cells were plated into 38-mm^2 wells of 96-well tissue culture plates. The cells were grown in DMEM supplemented with sodium pyruvate, essential amino acids, and 10% fetal bovine serum. After a 24-hour attachment period, the cells were refed with this medium (the negative control was refed with DMEM alone) or medium containing the ILK inhibitor QLT0267. After incubation for 3 days, the number of metabolically active cells was determined by MTT assay: the conversion of MTT to formazan was measured by a 96-well microtiter plate reader (MR-5000; Dynatech, Inc., Chantilly, VA) at an

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6 D. Morrison, personal communication.
absorbance of 570 nm. Growth inhibition was calculated with the following formula: cytostasis \(\% = \frac{A - (A/B)}{B} \times 100\), where \(A\) is the absorbance of treated cells and \(B\) is that of control cells.

**Measurement of Apoptosis**

Cells were plated at a density of \(5 \times 10^5\) in 38-mm² six-well plates (Costar, Cambridge, MA) and incubated at 37°C for 24 hours before treatment with QLT0267. Seventy-two hours later, the extent of cell death was determined by propidium iodide staining of hypodiploid DNA: \(3 \times 10^5\) cells were resuspended in Nicoletti buffer (50 μg/mL Sigma Chemical, St. Louis, MO), 0.1% sodium citrate, 0.1% Triton X-100, and 1 mg/mL of RNase (Roche, Basel, Switzerland) in PBS and then analyzed by fluorescence-activated cell sorter analysis (FACScan, Becton Dickinson, Mountain View, CA). The fraction of cells with sub-G₁ DNA content was assessed using the Lysis program (Becton Dickinson). The percentage of specific apoptosis was calculated by subtracting the percentage of spontaneous apoptosis of the relevant controls from the total percentage of apoptosis.

**Western Immunoblotting**

Cells were grown in 10-cm culture flasks (Corning, Life Sciences, Inc., Big Flats, NY) in DMEM 10% fetal bovine serum. After the cells reached 70% to 80% confluency, the medium was discarded and adherent cells were washed twice with ice-cold PBS. The cells were then solubilized in lysis buffer [1× concentration: 20 mmol/L Tris (pH 7.5), 1,50 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 μg/mL of leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride] for 15 minutes on ice.

Lysates were clarified by centrifugation at 10,000 g for 15 minutes at 4°C. Equal amounts of protein (30–50 μg) were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Billerica, MA), which was blocked with 1% milk in TBS-T for 30 to 45 minutes at room temperature. Afterwards, the membrane was washed thrice with TBS-T for 10 minutes each, the corresponding secondary antibody was added at the proper dilution and left overnight at 4°C. After the membrane was washed thrice with TBS-T for 10 minutes each, the corresponding secondary antibody was added at the proper dilution for 1 hour at room temperature. Then, the membrane was washed thrice with TBS-T, and protein detection was carried out using horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (IgG) from Santa Cruz Biotechnology or anti-mouse IgG from Sigma Chemical and an enhanced chemiluminescence kit (Amersham Pharmacia, Little Chalfont Buckinghamshire, United Kingdom).

**ILK Kinase Assay**

Lysate (250 μg) was immunoprecipitated overnight with 1 μg of rabbit polyclonal anti-ILK antibody (Upstate Biotechnology) or 1 μg of polyclonal rabbit IgG (Santa Cruz Biotechnology) as a negative control. We used the nonradioactive AKT kinase kit from Cell Signaling Technology. In brief, 30 μL of protein A agarose beads were added to the immune complexes and rotated for 2 to 3 hours at 4°C. Afterwards, they were centrifuged for 30 seconds at 4°C, and the pellets were washed twice with 500 μL of lysis buffer and twice with 500 μL of kinase buffer [25 mmol/L Tris (pH 7.5), 5 mmol/L β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na₃VO₄, and 10 mmol/L MgCl₂].

For the cold kinase assay, the pellets were suspended in 40 μL of kinase buffer supplemented with 200 μmol/L ATP and 1 μg of GSK-3 fusion protein. After incubation at 30°C for 30 minutes, the reaction was terminated using 20 μL of 3× SDS sample buffer, mixed with a vortex mixer, and then spun in a microcentrifuge for 2 minutes. The supernatant was transferred to a new tube; the sample was boiled for 5 minutes and loaded (15–30 μL) onto 10% SDS-polyacrylamide gel. Phosphorylation of the substrate was detected by Western blot analysis with the anti-GSK-3 Ser²¹/⁹ antibody at 1:1,000 dilution (Cell Signaling Technology).

**Animals**

Eight- to 12-week-old male athymic nude C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD) and housed in a specific pathogen-free animal facility. The animals were fed irradiated mouse food and autoclaved reverse osmosis treated water. All animal procedures were done in accordance with a protocol approved by our institutional Animal Care and Use Committee. All mice were euthanized after 6 weeks by asphyxiation with carbon dioxide.

**Subcutaneous Xenografts to Assess In vivo ILK Expression**

Thyroid cancer cells were harvested from subconfluent cultures by trypsinization and washed. One million cells of DRO, Hth74, ARO, K4, and NPA187 were separately injected s.c. using a 30-gauge needle into the flanks of the athymic nude mice (10 mice per cell line). The mice were weighed and the tumors measured weekly using microcalipers until the mice were euthanized 2 weeks after the injection of tumor cells. For immunohistochemical and routine H&E staining, the tumor tissue was fixed in formalin and embedded in paraffin.

**Human Thyroid Tissue Arrays**

Thyroid tumor tissue arrays representative of the entire spectrum of benign and malignant neoplasms, including ATC constructed at the head and neck tissue care facility, were used to screen for epidermal growth factor receptor expression. The arrays represented 25 papillary carcinomas, 33 medullary carcinomas, 21 anaplastic carcinomas, 55 follicular carcinomas, 20 Hürthle carcinomas, and eight samples of normal nondiseased thyroid tissue. Two cores of each sample were placed differentially in the recipient block. Two pathologists scored these blindly and independently on a scale of 0 to 3. Whenever a discrepancy in scoring was noted, both pathologists reexamined the sample in question and a consensus was reached.

**Immunohistochemical Staining of Murine Tumor and Human Tissue Sections**

Antibody staining was done on 5-μm histologic sections of formalin-fixed, paraffin-embedded specimens. All
sections were deparaffinized and hydrated by exposure to the following: xylene for 3 minutes; 100% ethanol for 2 minutes, twice; 95% ethanol for 1 minute, twice; 80% ethanol for 1 minute; and PBS for 2 minutes, twice. Afterwards, antigen retrieval was done using Target retrieval solution (DakoTarget; DAKO Corp., Carpinteria, CA). After incubation with 0.3% peroxide in methanol and incubation with normal blocking serum for 30 minutes at room temperature, sections were incubated overnight at 4°C with primary anti-ILK antibody at 1:100 dilution (Upstate Biotechnology). Immunodetection was done with peroxidase-labeled secondary antibody diluted in blocking solution for 1 hour at room temperature using DAB as the chromogen. Positive controls for ILK immunostaining were formalin-fixed, paraffin-embedded surgical specimens of human prostate tissue. Negative controls included sections stained with nonspecific rabbit IgG at the same protein concentration as the primary anti-ILK antibody. All sections were counterstained with Gill’s hematoxylin.

**Tumor Xenograft Generation for the ILK Inhibitor Treatment Study**

To produce tumors, DRO cells were harvested from subconfluent cultures by a brief 2-minute exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum. The cells were washed once in serum-free medium and resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used for the injections. One million DRO cells were suspended in 0.1 mL of HBSS and injected s.c. into the right flank area of nude mice. After 7 days, all mice were examined. Forty mice with homogeneous tumors were identified and then randomized into four groups (n = 10 each): group 1 was the untreated control group, group 2 was the group treated with PTE vehicle only, group 3 was the group treated with QLT0267 (50 mg/kg) by oral gavage daily, and group 4 was the group treated with QLT0267 (100 mg/kg) by oral gavage daily. Tumor size was measured twice a week in each mouse with calipers. Tumor volume (in mm³) was calculated using the formula $V = \frac{A^2 \pi}{6}$, where $A$ is the length of the longest aspect of the tumor and $B$ is the length of the tumor perpendicular to $A$.

The mice were euthanized once they became moribund or lost >20% of their recorded initial body weight before succumbing to tumor progression. After the mice were euthanized, necropsies were done and the xenografted tumors were obtained. For immunohistochemical and routine H&E staining, half of the tumor tissue was fixed in formalin and embedded in paraffin. The other half was embedded in ornithine carbamyl transferase compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C.

**Immunohistochemical Detection of CD31/Platelet/Endothelial Cell Adhesion Molecule 1, ILK, p-AKT, and Total AKT**

Frozen tissues were sectioned into 8- to 10-μm slices and used for detection of AKT/p-AKT and CD31/platelet/endothelial cell adhesion molecule 1. The slices were mounted on positively charged Plus slides (Fisher Scientific, Pittsburgh, PA) and air-dried for 30 minutes; fixed in cold acetone (5 minutes), 1:1 acetone/chloroform (v/v; 5 minutes), and acetone (5 minutes); and then washed with PBS. Immunohistochemical procedures were done as described previously. Dilutions of primary antibodies were as follows: AKT, 1:100; p-AKT, 1:100; CD31/platelet/endothelial cell adhesion molecule 1, 1:400; and ILK, 1:100.

Peroxidase-conjugated secondary antibody was used for immunohistochemical analysis of CD31/platelet/endothelial cell adhesion molecule 1. A positive reaction for p-AKT was visualized by incubating the slides for 1 hour with a 1:600 dilution of conjugated secondary antibody. Bleaching of fluorescence was minimized by covering the slides with 90% glycerol and 10% PBS. A positive reaction was visualized by incubating the slides with stable 3,3′-diaminobenzidine for 10 to 20 minutes for identification of CD31/platelet/endothelial cell adhesion molecule 1. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 minute, and mounted with Universal Mount (Research Genetics, Huntsville, AL). Control samples that had not been exposed to primary antibody showed no specific staining.

**Staining for CD31/Terminal Deoxynucleotidyl Transferase – Mediated Nick-End Labeling and Double Immunofluorescence Assays**

For terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) and double immunofluorescence assays, frozen tissues were used. After being mounted on slides and fixation with acetone as described above, the frozen samples were washed thrice with PBS, incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 minutes at room temperature, and then incubated with a 1:400 dilution of rat anti-mouse CD31 monoclonal antibody (human cross-reactive) overnight at 4°C. After the samples were rinsed thrice with PBS for 3 minutes each, the slides were incubated for 1 hour at room temperature in the dark with a 1:600 dilution of secondary goat anti-rat antibody conjugated to Alexa Fluor 594 (red fluorescence). The samples were then washed thrice with PBS containing 0.1% Brij and once with PBS for 3 minutes.

**TUNEL Assay**

A TUNEL assay was done using an apoptosis detection kit (Promega, Madison, WI) with the following modifications: samples were fixed with 4% paraformaldehyde (methanol free) for 10 minutes at room temperature, washed twice with PBS for 5 minutes, and incubated with 0.2% Triton X-100 for 15 minutes at room temperature. After two 5-minute washes with PBS, the samples were incubated with equilibration buffer for 10 minutes at room temperature. The equilibration buffer was drained, and reaction buffer, which contained 44 μL of equilibration buffer, 5 μL of nucleotide mix, and 1 μL of terminal deoxynucleotidyl transferase (supplied in the kit), was added to the tissue sections and incubated in a humid
atmosphere at 37°C for 1 hour, avoiding exposure to light. The reaction was terminated by immersing the samples in 2X SSC for 15 minutes. Samples were then washed thrice for 5 minutes to remove unincorporated fluorescein-dUTP.

**Quantification of Mean Vascular Density and Apoptotic Endothelial Cells**

For quantification analysis, five slides were prepared for each group, and two areas were selected on each slide. To quantify the expression in the TUNEL assays, the number of positively stained cells and total cells were also counted in 10 random 0.159-mm² fields of tumor area at ×100 magnification, and the percentages of positively stained cells from among the total number of cells were calculated and compared.

Immunofluorescence microscopy was done using a Nikon Microphot-FX (Nikon, Inc., Garden City, NY) equipped with a HBO 100 mercury lamp and narrow band-pass filters to individually select for green, red, and blue fluorescence (Chroma Technology Corp., Brattleboro, VT). Images were captured using a cooled charged coupled device Hamamatsu 5810 camera (Hamamatsu Corp., Bridgewater, NJ) and Optimas Image Analysis software (Media Cybernetics, Silver Spring, MD). Stained sections were examined in a Nikon Microphot-FX microscope equipped with a three-chip charged coupled device color video camera (model DXC990, Sony Corp., Tokyo, Japan). Endothelial cells were identified by red fluorescence staining, and DNA fragmentation was detected by localized green fluorescence within the nuclei of apoptotic cells. Photomontages were prepared in a Sony digital color printer (model UPD7000).

To quantify mean vascular density (MVD), the areas containing the higher number of tumor-associated blood vessels were identified by scanning the tumor sections at low microscopic power (×40). Vessels that were completely stained with anti-CD31 antibodies were then counted in 10 random 0.159-mm² fields at ×100 magnification. Quantification of apoptotic endothelial cells was expressed as the average of the ratios of apoptotic endothelial cells to the total number of endothelial cells in 10 random 0.011-mm² fields at ×400 magnification.

**Statistical Methods**

Differences between various tumor types were analyzed for their correlation with ILK expression. To test for statistical significance between the intensities of ILK staining in human tissues, associations between categorical variables were assessed via cross-tabulation and Fisher's exact test. All computations were carried out on a personal computer using the Windows NT operating system (Microsoft, Redmond, WA) using StatXact 4.0 software.

To assess the differences in tumor volumes between the four groups of mice and to statistically quantitate the differences in TUNEL, MVD, and CD31-TUNEL staining, we used Wilcoxon’s test or the nonparametric Kruskal-Wallis test. The Kruskal-Wallis test was used to detect the difference between the groups on each observation day, and Wilcoxon’s test was used to assess the difference between each treatment and control group on each observation day. All statistical computations were done on a Dell 1,000-mHz personal computer using SAS statistical software (SAS Institute, Cary, NC). *Ps < 0.05* were considered statistically significant.

**Results**

**ILK Protein Expression Is Elevated in Human Thyroid Cancer Specimens**

After verifying the overexpression of ILK in thyroid cancer cell lines, we examined whether this observation translates to human thyroid cancer specimens. Using immunohistochemical techniques, we assessed the level of ILK protein expression in a panel of human thyroid...
tissue microarrays that contained normal thyroid tissue and specimens of various thyroid cancer subtypes. Although ILK protein expression was detected in most of the normal thyroid tissue sections (75% of slides), the intensity of staining was significantly higher in the tumor sections. The percentages of slides staining positively at 2+ and 3+ levels were as follows: normal (12.5%), papillary (12%), medullary (39.4%), follicular (27.3%), anaplastic (57%), and Hürthle cell carcinoma (77.8%; Fig. 1; Table 1). Both anaplastic and Hürthle cell (a subgroup of follicular) thyroid carcinomas stained significantly stronger than normal human thyroid tissue ($P = 0.044$ and 0.011, respectively).

ILK Protein Expression and Kinase Activity Are Elevated in a Panel of Thyroid Cancer Cell Lines

Using Western blotting, we assessed ILK protein expression in one papillary and five anaplastic thyroid cancer cell lines. ILK protein was expressed in all cell lines and was highly so in the DRO, K4, KAT-4, and ARO anaplastic cell lines. The anaplastic (Hth74) thyroid cancer and the papillary (NPA187) cell lines showed lower expression of the ILK protein (Fig. 2A). In an in vitro kinase assay, all six thyroid cancer cell lines showed high levels of ILK kinase activity (Fig. 2B).

### Table 1. Immunohistochemical analysis of ILK expression in a human papillary cancer tissue microarray

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>No. tumors</th>
<th>Staining intensity</th>
<th>% Specimens staining positive for ILK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 1+ 2+ 3+</td>
<td>2+ and 3+</td>
</tr>
<tr>
<td>Normal</td>
<td>8</td>
<td>2 5 0 1</td>
<td>12.5 75</td>
</tr>
<tr>
<td>Papillary</td>
<td>25</td>
<td>15 7 3 0</td>
<td>12 40</td>
</tr>
<tr>
<td>Medullary</td>
<td>33</td>
<td>10 10 7 6</td>
<td>39.4 71.4</td>
</tr>
<tr>
<td>Follicular</td>
<td>55</td>
<td>32 8 8 7</td>
<td>27.3 41.8</td>
</tr>
<tr>
<td>Anaplastic</td>
<td>21</td>
<td>4 5 4 8</td>
<td>57* 81</td>
</tr>
<tr>
<td>Hürthle cell</td>
<td>20</td>
<td>2 4 5 9</td>
<td>77.8* 81.8</td>
</tr>
<tr>
<td>Total</td>
<td>162</td>
<td>65 39 27 31</td>
<td>29 81</td>
</tr>
</tbody>
</table>

*Statistically significant compared with normal ($P = 0.044$, anaplastic; $P = 0.011$, Hürthle; Fisher’s exact test).
ILK Is Overexpressed in Thyroid Cancer Xenografts
To determine whether ILK expression in the previously tested thyroid cancer cell lines exceeds that in normal thyroid cells, we s.c. injected one million cells each of NPA187, ARO, DRO, K4, and Hth74 cell lines in nude mice. On immunohistochemical analysis, ILK was overexpressed in all tested cell line xenografts compared with normal thyroid tissue (Fig. 2C).

QLT0267 Inhibits Growth of Thyroid Cancer Cells

**In vitro**
QLT0267 was developed through the optimization of a lead compound identified in the high-throughput screening of a rationally designed small molecule library against the target ILK. The initial lead compound was found to be a submicromolar inhibitor of the phosphotransferase activity of ILK towards a specific peptide substrate. An intensive medicinal chemistry effort around this lead compound has identified many structure/activity relationships and allowed for the identification of a bioactive core of the molecule. Further optimization of this molecular core to enhance both *in vitro* enzyme and cell-based potencies and to improve the pharmacokinetic and pharmacodynamic properties of this class of compounds has lead to the second-generation compound QLT0267. QLT0267 has been shown to inhibit the kinase activity of ILK in cell-free assay at 26 nmol/L and preliminary experiments suggest that it has ~1,000-fold selectivity over other kinases tested under similar conditions, including CK2, CSK, DNA-PK, PIM1, protein kinase B or AKT kinase, and PKC; ~100-fold selectivity over extracellular signal-regulated kinase 1, GSK3β, LCK, PKA, p70S6K, and RSK1 (QLT). Of those tested, CDK1, CDK2, and CDK5 showed the greatest inhibition by QLT0267 but the selectivity window is still at least 10-fold. In an MTT assay, one papillary (NPA187) and two anaplastic cell lines (K4, a low expressor of ILK protein and DRO, a high expresser of ILK protein) were subjected to increasing concentrations of QLT0267 for 72 hours. QLT0267 inhibited cell growth in all cell lines with an IC50 of ~3 μmol/L (Fig. 3A).

QLT0267 Induces Apoptosis in Thyroid Cancer Cells

**In vitro**
Using an apoptosis assay based on flow cytometry, we assessed the ability of QLT0267 to induce apoptosis in the DRO cell line. When cells were subjected to increasing concentrations of QLT0267 for 48 hours, 10% of them underwent apoptosis at a dose as low as 3 μmol/L, and a maximum of about 69% reached cell death at ~12 μmol/L (Fig. 3B). Furthermore, when the NPA187, DRO, and K4 cell lines were subjected to a 3 μmol/L concentration of QLT0267 for 24 and 48 hours, all three cell lines underwent apoptosis, as evidenced by the induction of DNA fragmentation (Fig. 3C).

Figure 3. Effect of QLT0267 on cell growth, proliferation, and induction of apoptosis in thyroid cancer cell lines. **A**, results of MTT assay on K4, DRO, and NPA187 thyroid cancer cell lines treated with QLT0267 showing that reduction of cellular proliferation was first seen at a QLT0267 concentration of 1 μmol/L, and the IC50 was 3 μmol/L after treatment for 72 h. Near-maximal growth inhibition was seen at a concentration of 15 μmol/L. **B**, propidium iodide apoptosis assay on the DRO cell line in the presence of 2% serum and treated for 48 h with increasing concentrations of QLT0267 showing that apoptosis induction was induced at the 3 μmol/L concentration (10% cell death) and reached its peak (69% cell death) at a concentration of about 12 μmol/L. **C**, results of DNA fragmentation assay on all three thyroid cancer cell lines in the presence of 2% serum and treated for various periods with 3 μmol/L of QLT0267. Representative of three independent experiments.
QLT0267 Reduces ILK Kinase Activity in Thyroid Cancer In vitro

In an in vitro kinase assay, 3 μmol/L QLT0267 reduced ILK kinase activity in all five tested anaplastic thyroid cancer cell lines. In four of these cell lines (ARO, DRO, Hth74, and K4), ILK in vitro kinase activity was greatly diminished compared with that of the respective DMSO-treated cells (Fig. 4A).

QLT0267 Inhibits Epidermal Growth Factor – Induced Phosphorylation of AKT in Thyroid Cancer Cells In vitro

When Hth74 cells were starved of serum overnight, treated with as little as 1 μmol/L QLT0267 for a prolonged period of time, and then stimulated with epidermal growth factor for 15 minutes, epidermal growth factor–induced phosphorylation of AKT, as measured by Western blotting, was inhibited (Fig. 4B). There was a time dependent inhibition of the phosphorylation of AKT with a maximal inhibition reached at 6 hours.

QLT0267 Induces Tumor Growth Inhibition of DRO Xenografts In vivo

To assess the effects of QLT0267 on tumor growth, we injected DRO cells s.c. into athymic nude mice. All mice were killed on day 16 after the start of treatment because the mice in the untreated control and vehicle-only groups had large tumors or had become moribund owing to the tumor burden. As shown in Fig. 5, the tumor volumes in both groups of treated mice (50 and 100 mg/kg) were significantly lower than the tumor volumes in both the untreated control and vehicle-control groups (P < 0.005 at d 10 and P < 0.001 at day 16). Treatment with QLT0267 was well tolerated, as determined by the maintenance of body weight of mice in both treated groups (data not shown).

QLT0267 Inhibits p-AKT and Induces Apoptosis In vivo

To evaluate the mechanism by which QLT0267 works in vivo, we analyzed the DRO tumors using immunohistochemical and immunofluorescence analyses for markers of survival and apoptosis. No differences in the level of ILK staining were detected in the four groups of mice. Immunofluorescence analysis revealed that the levels of total AKT in tumors were similar in the four groups. However, the levels of expression of phosphorylated AKT (Ser473) in the tumor tissues between the QLT0267-treated groups and the two control groups differed greatly. As predicted, phosphorylated AKT was expressed in the two control groups but rarely expressed in the two treated groups (Fig. 6A) thus confirming that QLT0267 treatment inhibits the phosphorylation of AKT in vivo.

To assess the effect of QLT0267 on the induction of apoptosis in vivo, we used the immunofluorescence TUNEL assay, which revealed that the apoptotic fraction in the tumor specimens was greater for mice treated with QLT0267 than it was for the two control groups of mice (Fig. 6B). The numbers of TUNEL-positive cells per high-power field in the untreated control and vehicle-control groups were 3.30 ± 0.82 and 3.76 ± 0.88, respectively, compared with 23.87 ± 2.23 in the group treated with 50 mg/kg of QLT0267 and 35.97 ± 7.89 in the group treated with 100 mg/kg of QLT0267 (P < 0.001 for both when compared with control, Fig. 6C). These in vivo data are consistent with the in vitro results measured by the MTT and propidium iodide apoptosis assays.

QLT0267 Reduces MVD and Induces Tumor-Associated Endothelial Cell Apoptosis

To determine whether QLT0267 has an antiangiogenic effect, the MVD was measured by staining the tumor specimen with antibodies against CD31. Treatment with QLT0267 significantly reduced the number of blood vessels from 7.78 ± 1.85 in the tumors from untreated control mice and 7.53 ± 1.1 in those from the vehicle-treated mice to 5.68 ± 1.37 in the tumors from mice treated with 50 mg/kg of QLT0267 and 35.97 ± 7.89 in the group treated with 100 mg/kg of QLT0267 (P < 0.001 for both when compared with control, Fig. 6C). These in vivo data are consistent with the in vitro results measured by the MTT and propidium iodide apoptosis assays.

Finally, we investigated whether QLT0267 induces apoptosis of tumor-associated endothelial cells. Endothelial cells of tumors resulting from injection of DRO cells underwent apoptosis, as shown by double staining for...
expression has been linked to the progression of tumor invasion and metastasis (25). In addition, ILK overexpression is closely correlated with gastric cancer survival rate patients with prostate cancer (24). Similarly, expression is inversely related to the 5-year patient increases with prostate tumor grade, and enhanced ILK of tumors (23–25, 31). For example, ILK expression previous studies on ILK overexpression in various kinds associated endothelial cells. induction of apoptosis in both tumor cells and tumor-inhibition of angiogenesis (reduction in MVD), and to a reduction in survival signals (reduction in p-AKT), volume by day 16. This tumor reduction was attributed mental doses of QLT0267 significantly reduced tumor mice bearing DRO tumor xenografts with two experi- and induction of apoptosis. Furthermore, treatment of lines (NPA187, DRO, and K4) led to growth inhibition new ILK inhibitor, QLT0267, in three thyroid cancer cell lines (NPA187, DRO, and Hth74) showed a significant reduction in tumor volume compared with the control or vehicle-only mice (P < 0.005 at d 10 and P < 0.001 at day 16).

CD31 and TUNEL (Fig. 6B). The percentage of apoptotic endothelial cells was significantly higher in DRO xenografts harvested 16 days after the initiation of treatment with QLT0267 than in those from the untreated control and vehicle-control groups (P < 0.01; Fig. 6C).

Discussion
In this study, we showed that a panel of thyroid cancer cell lines had elevated ILK protein expression and kinase activity. We also found that when these cell lines were injected s.c. into athymic nude mice, they retained their high level of ILK protein expression compared with that in the normal thyroid gland of nude mice. Furthermore, the intensity of ILK protein expression in a panel of human anaplastic thyroid cancer specimens was higher than it was in normal thyroid tissue. Treatment with a new ILK inhibitor, QLT0267, in three thyroid cancer cell lines (NPA187, DRO, and K4) led to growth inhibition and induction of apoptosis. Furthermore, treatment of mice bearing DRO tumor xenografts with two experimental doses of QLT0267 significantly reduced tumor volume by day 16. This tumor reduction was attributed to a reduction in survival signals (reduction in p-AKT), inhibition of angiogenesis (reduction in MVD), and induction of apoptosis in both tumor cells and tumor-associated endothelial cells.

Most of these data are consistent with those from previous studies on ILK overexpression in various kinds of tumors (23–25, 31). For example, ILK expression increases with prostate tumor grade, and enhanced ILK expression is inversely related to the 5-year patient survival rate patients with prostate cancer (24). Similarly, ILK expression is closely correlated with gastric cancer invasion and metastasis (25). In addition, ILK overexpression has been linked to the progression of tumor grade in ovarian cancer (31). In an ongoing study, we found that ILK protein expression and activity are highly elevated in squamous cell carcinoma of the head and neck.7

Although ILK protein expression in the tested thyroid cancer cell lines was related to their respective tumor xenografts in mice, NPA187 and Hth74 cell lines showed a much lower expression of ILK in vivo. This finding may point to the fact that ILK protein expression in vivo is controlled by various factors that are not present in tissue culture (i.e., interactions with various components of the extracellular matrix).

Among the limitations of this study was the use of a limited number (n = 5) of anaplastic thyroid cancer cell lines. Despite this small number, the cell lines we used are among the most well established in the literature (30, 32, 33). We are currently working with a larger panel of cell lines to verify our findings.

Although our in vitro data indicated that the ILK inhibitor has direct antitumor activity, our immunohistochemical analysis of tumors that responded to ILK treatment in nude mice indicated that ILK also could induce apoptosis in the tumor vasculature. Our findings of decreased MVD and tumor endothelial cell apoptosis substantiate the ability of ILK to induce apoptosis in the tumor vasculature but do not explain whether the ILK inhibitor acts directly on the tumor endothelium or indirectly by changing the pattern of endothelial growth factors elaborated by the tumor cells.

Other investigators have reported finding this link between ILK and regulation of angiogenesis. Tan et al. (30) showed that ILK plays a pivotal role in mediating vascular endothelial growth factor signaling in endothelial cells and induces hypoxia-inducible factor-1α-dependent vascular endothelial growth factor expression in prostate cancer cells. Our preliminary data showed that our anaplastic thyroid cancer cell lines produced varying levels of vascular endothelial growth factor (data not shown). In our animal experiments reported here, we detected no change in the levels of vascular endothelial growth factor expression, as determined immunohistochemically, between QLT0267-treated groups and the control groups.

To our knowledge, little has been published on the pharmacologic inhibition of ILK (i.e., only a few studies on the use of the specific ILK inhibitors KP-SD-1 and KP 392; refs. 16, 21, 34, 35). More recently, the efficacy of a similar ILK inhibitor QLT0254 on pancreatic carcinoma growth in vitro and in vivo has been established (36). We have tested four different ILK inhibitors on a panel of squamous cell carcinoma of the head and neck cell lines and found that QLT0267 was the most effective inducer of growth arrest and apoptosis.8 QLT0267 has proven its efficacy in reducing cellular growth and inducing apoptosis in various forms of thyroid cancer cells.

7 Unpublished data.
8 Younes et al., in preparation.
Figure 6. Immunohistochemical analysis of ILK, AKT, p-AKT, CD31, TUNEL, and CD31/TUNEL staining in the four mice groups. A, after 16 d of treatment with QLT0267, tumors were processed for immunohistochemical analysis. Tumors from untreated control and vehicle-control mice expressed p-AKT, whereas those from mice in both treatment groups lost their p-AKT expression. No difference was found in total AKT expression among the four groups. ILK expression remained unaltered in tumors from the four groups. B, in the control and vehicle groups, DKO tumors showed an increase in the number of blood vessels (brown staining), as marked by CD31 staining. Both treatment groups had tumors that showed a reduced number of blood vessels. Apoptosis as marked by TUNEL staining was markedly elevated in both treatment groups (green staining) compared with control and vehicle tumors (B). When colocalizing for both CD31-positive endothelial cells and apoptotic cells, more endothelial cells were found to undergo apoptosis in the 100-mg/kg treatment arm of the experiment than in the control, vehicle, and the 50-mg/kg treatment arms. C, quantitative analysis of the immunohistochemistry data. i, the number of TUNEL+ cells per unit area in the control and mice receiving the vehicle were 15.83 and 23.75, respectively, compared with 34.06 in the group given 50 mg/kg QLT0267 and 73.51 in the group given 100 mg/kg QLT0267 (P < 0.001). ii, MVD of the tumor was decreased from 7.78 in the control tumors and 7.53 in the vehicle tumors to 5.68 in the 50 mg/kg KP74728-treated tumors (P < 0.05) and 4.78 in the 100 mg/kg KP74728-treated tumors (P < 0.001). iii, numbers of tumor-associated endothelial cells undergoing apoptosis were elevated in the 100 mg/kg treatment arm (73.51; P < 0.01) compared with the control and vehicle groups (15.83 and 23.75, respectively).

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In summary, our findings shed some light on the biology of anaplastic thyroid cancer, which is rare and nearly always fatal, and on the potential role that ILK might play in mediating tumorigenesis by regulating tumor cell growth and angiogenesis. The use of the novel ILK inhibitor QLT0267 on the tested cell lines was associated with cell growth arrest, induction of apoptosis in vitro, and reduction of tumor volume in vivo.

References
Integrin-linked kinase is a potential therapeutic target for anaplastic thyroid cancer

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