SKI-606, a Src inhibitor, reduces tumor growth, invasion, and distant metastasis in a mouse model of thyroid cancer

Won Gu Kim\textsuperscript{1}, Celine J Guigon\textsuperscript{1*}, Laura Fozzatti\textsuperscript{1}, Jeong Won Park\textsuperscript{1}, Changxue Lu\textsuperscript{1}, Mark C Willingham\textsuperscript{2}, and Sheue-yann Cheng\textsuperscript{1}

\textsuperscript{1}Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA, \textsuperscript{2}Department of Pathology, Wake Forest University, Winston-Salem, NC, USA

\textsuperscript{*}Current address: Université Paris Diderot, Sorbonne Paris Cité, Equipe Physiologie de l’Axe Gonadotrope, Unité Biologie Fonctionnelle et Adaptative, Centre National de la recherche Scientifique, Paris, France

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Correspondence
Dr. Sheue-yann Cheng
Gene regulation section, Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute
37 Convent Dr, Room 5128, Bethesda, MD 20892-4264, USA
E-mail: chengs@mail.nih.gov

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Abstract

Purpose: Src is over-expressed or hyper-activated in a variety of human cancers including thyroid carcinoma. Src is a central mediator in multiple signaling pathways that are important in oncogenesis and cancer progression. In this study, we evaluated the effects of a Src inhibitor, SKI-606 (bosutinib), in a spontaneous metastatic thyroid cancer model with constitutively activated Src (Thr{sup PV}/Pten{sup +/−} mice).

Experimental Design: Thr{sup PV}/Pten{sup +/−} mice were treated with SKI-606 or vehicle controls, beginning at 6 weeks of age until the mice succumbed to thyroid cancer. We assessed the effects of SKI-606 on thyroid cancer progression and analyzed the impact of SKI-606 on aberrant Src-mediated signaling.

Results: SKI-606 effectively inhibited aberrant activation of Src and its downstream targets to markedly inhibit the growth of thyroid tumor, thereby prolonging the survival of treated mice. While Src inhibition did not induce cell apoptosis, it decreased cell proliferation by affecting the expression of key regulators of cell cycle progression. Importantly, SKI-606 dramatically prevented de-differentiation, vascular invasion, and lung metastasis of thyroid cancer cells. These responses were mediated by down-regulation of mitogen-activated protein kinase pathways and inhibition of the epithelial-mesenchymal transition.

Conclusions: Our findings suggest that Src is critical in the progression of thyroid cancer, making oral SKI-606 a promising treatment strategy for refractory thyroid cancer.
Translational Relevance

About 30% of recurrent or metastatic differentiated thyroid cancers show de-differentiation of malignant cells and subsequently become resistant to radioiodine therapy with poor prognosis. Although some kinase inhibitors have been in trials for this subgroup of patients, no effective therapy was approved for radio-refractory differentiated thyroid cancer. Src is frequently over-activated in human thyroid cancer and is a central mediator in multiple signaling pathways critical in carcinogenesis. Our studies demonstrated that inhibition of Src by SKI-606 decreased tumor growth, inhibited de-differentiation and blocked distant metastasis. The present preclinical studies indicate that the Src inhibitors are potentially effective strategies for single-drug therapy or in combination with radioiodine therapy to treat refractory thyroid cancer.
Introduction

The incidence of thyroid cancer, the most common malignancy in the endocrine organs has greatly increased in the past two decades around the world (1). In the United States, it is estimated that 48,020 new cases of thyroid cancer will be diagnosed in 2011, and the age-adjusted incidence increased by 6.4% between 1997 and 2008 (2). Approximately 95% of all thyroid cancer is differentiated thyroid cancer, which has a good prognosis after initial treatment regimens, including thyroid surgery, adjuvant radioiodine, and thyroxine suppression of thyroid-stimulating hormone (TSH). However, distant metastases at the time of diagnosis are present in 5% of patients, and recurrent disease occurs in another 10-15%. Approximately half of these cases can be cured with additional surgery or additional radioiodine therapy, but the other half become radio-refractory and those patients have a poor survival rate (3-5). Currently, no effective therapy exists for this subgroup of patients.

A number of multi-targeted kinase inhibitors designed for patients with advanced or progressive metastatic thyroid cancers have entered clinical trials. Most of these agents have targeted angiogenesis primarily, and the activity they have in common is that of inhibiting the vascular endothelial growth factor receptor (4, 6). However, none of these kinase inhibitors has yet been shown to improve survival for thyroid cancer patients. The low rate of partial responses, the absence of complete responses, and the emergence of resistance in all of the various monotherapy trials underscore the need either to develop more effective single agents or to identify rational combinations of therapeutic targets that have synergistic effectiveness (6).

Src plays critical roles in cell proliferation, survival, motility, migration, cell-matrix adhesion dynamics, and regulation of cytoskeleton, via multiple downstream signaling pathways including mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K), and focal adhesion kinase (FAK) (7-9). Src family kinases are over-
expressed or hyper-activated in human neoplasms including breast, colorectal, prostate, pancreas, head and neck, and lung, as well as thyroid carcinoma. Aberrant activation of Src is highly associated with the aggressive invasiveness of thyroid carcinoma (10-12). The Src inhibitors—dasatinib, saracatinib, and SKI-606 (bosutinib)—are in phase II clinical trials for treatment of metastatic breast and prostate cancer (7, 13). SKI-606, a multi-kinase inhibitor originally identified as a Src and Abl kinase inhibitor, is effective in vitro on chronic myeloid leukemia cells and breast and colorectal cancer cells; it is also effective in multiple xenograft tumor models (14-19). However, the effects of SKI-606 in patients with thyroid cancer have not yet been reported.

The development of a mouse model of thyroid cancer, the \( \text{Thrb}^{PV/PV} \) mouse, has provided a useful tool to elucidate the molecular basis of thyroid carcinogenesis (20, 21). As \( \text{Thrb}^{PV/PV} \) mice age, they spontaneously develop follicular thyroid carcinoma similar to human thyroid cancer with a pathological progression from hyperplasia to capsular invasion, vascular invasion, and eventually metastasis (20). We have recently shown that Src kinase pathway is activated to promote thyroid carcinogenesis of \( \text{Thrb}^{PV/PV} \) mice (22). The \( \text{Thrb}^{PV/PV} \text{Pten}^{+/−} \) mouse model was created by introducing haploid deficiency of the silencing of the tumor suppressor gene \( \text{Pten} \) (phosphatase and tensin homologue deleted from chromosome 10) into \( \text{Thrb}^{PV/PV} \) mice. PTEN deficiency further exacerbates the over-activated PI3K-AKT signaling, leading to more aggressive cancer phenotype with decreased survival and increased distant metastasis, making it useful for preclinical studies (22, 23).

The aim of the present study was to evaluate the effect of inhibiting Src activity by SKI-606 in the \( \text{Thrb}^{PV/PV} \text{Pten}^{+/−} \) mouse model. This spontaneous metastatic thyroid cancer model is ideal for assessing the effect of SKI-606 on cancer progression from early capsular invasion to late pulmonary metastasis. The effect of Src inhibition could be assessed in a whole-animal context because many functions of the Src family
kinases in multiple signaling pathways are integrated into an intact immune system and microenvironment (7, 8, 24).

Our studies demonstrated that SKI-606 treatment inhibited not only thyroid tumor growth but also distant metastasis of thyroid cancer. SKI-606 reduced tumor growth by inhibiting cell proliferation and preventing de-differentiation of tumor cells. These responses were accompanied by down regulation of MAPK pathways and inhibition of epithelial-mesenchymal transition (EMT). These findings from this preclinical study indicate that the Src inhibitors are potentially effective strategies for the treatment of refractory thyroid cancer.
Materials and Methods

Animals and treatment

The National Cancer Institute Animal Care and Use Committee approved the protocols for animal care and handling in the present study. Mice harboring the ThrhPV gene (ThrthPV/PV mice) were prepared via homologous recombination, and genotyping was carried out using the polymerase chain reaction method, as previously described (20). Pten+/− mice were kindly provided by Dr. Ramon Parsons (Columbia University, New York, NY). ThrhPV/PV Pten+/− mice were obtained by crossing Pten+/− mice with ThrhPV/PV mice, followed by crossing ThrhPV/+ Pten+/− with ThrhPV/+ Pten+/+ mice. SKI-606 (LC Laboratories, Woburn, MA) was dissolved in 0.5% methocel/0.4% Tween 80 (Sigma-Aldrich, ST. Louis, MO) in water and administered by oral gavage 5 times a week at a dose of 150 mg/kg body weight (14, 18) starting at the age of 6 weeks. Mice were monitored until they became moribund with rapid weight loss, hunched posture, and labored breathing. The thyroids and lungs were dissected after mice were euthanized for weighing, histological analysis, and biochemical studies.

Hormone assay

Serum levels of total T4 was determined by using a GammaCoat T4 assay RIA kit (DiaSorin, Stillwater, MN) as previously described (21). Serum TSH levels were measured as previously described (25).

Histopathological analysis

Thyroid glands and lungs were dissected and fixed in 10% neutral buffered formalin (Sigma-Aldrich), and subsequently embedded in paraffin. Five-micrometer-thick sections were prepared and stained with hematoxylin and eosin (H&E). For each animal, single random sections of thyroid and lung were examined. For thyroids, morphological
evidence of hyperplasia, capsular invasion, and vascular invasion was routinely examined in that single section. The presence of a single microscopic focus of metastatic follicular carcinoma in the lung was counted as a metastatic lesion in that animal.

Immunohistochemistry was performed as previously described with some modifications (26). For the antigen retrieval step, slides were heated in 0.05% citraconic anhydride solution (Sigma-Aldrich) (pH 7.4) at 98°C for 60 min followed by treatment with rabbit anti-Ki67 (1:300 dilution, NeoMarker; Thermo Scientific, Fremont, CA) at 4°C overnight. The antigen signals were detected by treatment with the peroxidase substrate diaminobenzidine followed by counterstaining with Gill’s hematoxylin.

Western blot analysis

Preparation of whole-cell lysates from thyroid glands has been described previously (27). The protein sample (20-30 μg) was loaded and separated by sodium dodecyl sulfated-polyacrylamide gel electrophoresis. After electrophoresis, the protein was electotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA). The antibodies p-Src (1:1000 dilution), total Src (1:1000 dilution), total FAK (1:500 dilution), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000 dilution), p-Rb (1:500 dilution), total Rb (1:250 dilution), CDK4 (1:1000 dilution), CDK6 (1:1000 dilution), Bad (1:500 dilution), cleaved caspase-3 (1:500 dilution), cleaved PARP (1:500 dilution), p-ERK (1:1000 dilution), total ERK (1:1000 dilution), p-p38 MAPK (1:1000 dilution), total p38 MAPK (1:1000 dilution), p-JNK (1:1000 dilution), total JNK (1:1000 dilution), vimentin (1:1000 dilution), and slug (1:1000 dilution) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for cyclin B1 (1:500 dilution), cyclin D1 (1:300 dilution), cyclin E (1:500 dilution), E2F1 (1:200 dilution), Bax (1:200
dilution), MMP-2 (1:200 dilution), MMP-9 (1:200 dilution), and E-cadherin (1:250 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for p-FAK (1:500 dilution) was from BioSource (Invitrogen, Carlsbad, CA). Antibody for Bcl-XL (1:1000 dilution) was purchased from NeoMarker (Thermo Scientific, Fremont, CA). The blots were stripped with Re-Blot Plus (Chemicon, Temecula, CA) and reprobed with rabbit polyclonal antibodies to GAPDH. Band intensities were quantified by using NIH IMAGE software (ImageJ 1.34s; Wayne Rasband, NIH).

**Quantitative real-time reverse transcriptase-PCR (RT-PCR)**

Total RNA from thyroid tumors were extracted by TRIzol (Invitrogen, Carlsbad, CA), followed by RNase-free DNase treatment (Qiagen, Valencia, CA) and was purified as described (RNeasy Mini Kit, Qiagen). One-step RT-PCR reactions were performed with 200 ng of total RNA using a QuantiTect SYBR green RT-PCR kit (Qiagen) in a Roche LightCycler PCR instrument (Roche, Indianapolis, IN) following the manufacturer's instructions. Specific primers were as followed: MMP-2 (F1910): 5'-CAG GGA ATG AGT ACT GGG TCT ATT-3'; MMP-2 (R2030): 5'-ACT CCA GTT AAA GGC AGC ATC TAC-3'; MMP-9 (F2319): 5'-AAT CTC TTC TAG AGA CTG GGA AGG AG-3'; MMP-9 (R2445): 5'-AGC TGA TTG ACT AAA GTA GCT GGA-3'; GAPDH (F681): 5'-ACA TCA TCC CTG CAT CCA CT-3'; GAPDH (R904): 5'-GTC CTC AGT GTA GCC CAA G-3'. The reaction conditions were 50°C for 20 min; 95°C for 15 min; 40 cycles of 94°C for 15s, 60°C for 25s, 72°C for 25s; and 65-95°C with a heating rate of 0.1°C/s and a cooling step to 40°C.

**Statistical analysis**
Data are presented as median/range or mean ± standard deviations, accordingly. Mann-Whitney U-test or Student's t-test was used to compare continuous variables accordingly. The Kaplan-Meier method with log-rank test was used to compare survival in each treatment group. P-values were two-sided throughout, p<0.05 was considered statistically significant. Data were analyzed using SPSS statistics 19.0 (SPSS Inc., Chicago, IL, USA). GraphPad PRISM 4.0a (GraphPad Software, San Diego, CA, USA) was used to draw graphs.
Results

Src inhibitor, SKI-606, significantly increases survival of $Thr b^{PV/PV} Pten^{+/−}$ mice by inhibition of thyroid tumor growth

Previously, we reported that the Src kinase pathway is activated in the thyroid of $Thr b^{PV/PV}$ mice (26). To evaluate whether Src kinase is also activated during thyroid carcinogenesis of $Thr b^{PV/PV} Pten^{+/−}$ mice, we compared the protein levels of phosphorylated Src (p-Src), total Src, phosphorylated FAK (p-FAK), and total FAK in wild-type (WT) and $Thr b^{PV/PV} Pten^{+/−}$ mice (Figure 1A). Src was highly activated as evidenced by markedly elevated phosphorylation of Src in the thyroids of $Thr b^{PV/PV} Pten^{+/−}$ mice as compare with WT thyroids (compare lanes 4-6 to lanes 1-3, Figure 1Aa) without affecting total Src protein levels (Figure 1Ab). FAK is a tyrosine kinase which is a critical downstream effector of Src signaling, affecting in cell cycle progression and survival as well as in adhesion and migration (8). Activated FAK was also evident by the increased phosphorylation of FAK in $Thr b^{PV/PV} Pten^{+/−}$ mice as compared with WT thyroids (compare lanes 4-6 with 1-3, Figure 1Ac). There were no apparent changes in total FAK protein levels in $Thr b^{PV/PV} Pten^{+/−}$ mice as compared with WT thyroids (compare lanes 4-6 with 1-3, Figure 1Ad). These findings suggested that the Src-FAK pathway is a potential target for treatment of thyroid cancer. Therefore, we evaluated the effect of the Src inhibitor, SKI-606, on thyroid carcinogenesis of $Thr b^{PV/PV} Pten^{+/−}$ mice.

To evaluate the extent of Src inhibition by SKI-606, p-Src (Y416) and total Src levels were analyzed by western blotting. As shown in Figure 1Ba, SKI-606 treatment inhibited the activity of Src by decreased p-Src (Y416) as compared with vehicle-only controls. Moreover, the activity of its downstream effector, FAK, was also inhibited by decreased phosphorylation of FAK (Y397) (Figure 1Bc) without changing the total FAK protein levels (Figure 1Bd).
To determine the effects of SKI-606 on thyroid carcinogenesis, we compared the survival of \( \text{Thrb}^{\text{PV/PV}} \text{Pten}^{\text{+/+}} \) mice with or without treatment with the inhibitor. Treatment of mice began at 6 weeks of age and continued until they became moribund with signs of palpable tumor, labored breathing, hunched posture, and rapid weight loss. In moribund mice, trachea was compressed due to the enlargement of thyroid tumor as previously reported (22, 23). Survival analysis over a period of 7 months (Figure 1C) showed that mice treated with SKI-606 (n=11) survival longer with an increase in 50% survival age of 1.2 months as compared with untreated mice (n=15, \( p<0.001 \)).

Thyroids of sacrificed \( \text{Thrb}^{\text{PV/PV}} \text{Pten}^{\text{+/+}} \) mice were dissected and expressed as the ratios of thyroid to the body weight. As shown in Figure 1D, thyroid weights of SKI-606-treated mice (n=11) were significantly lower than that of vehicle-treated mice (n=15, \( p=0.02 \)). There was no significant difference in total body weight between the mice treated with or without SKI-606 at the time of sacrifice (data not shown). These data suggested that the increased survival in SKI-606-treated mice was associated with a substantial decrease in thyroid weight as compared with vehicle-treated mice, indicating the effectiveness of SKI-606 in reducing tumor growth, leading to increased survival.

**SKI-606 inhibits vascular invasion and distant metastasis of thyroid cancer in \( \text{Thrb}^{\text{PV/PV}} \text{Pten}^{\text{+/+}} \) mice**

We further investigated whether SKI-606 treatment could prevent invasiveness and distant metastasis of thyroid cancer in \( \text{Thrb}^{\text{PV/PV}} \text{Pten}^{\text{+/+}} \) mice. As shown in Figure 1E, vascular invasion of thyroid cancer was significantly decreased in SKI-606-treated \( \text{Thrb}^{\text{PV/PV}} \text{Pten}^{\text{+/+}} \) mice as compared with vehicle-treated mice (\( p<0.001 \)). The development of lung metastasis was also significantly reduced in the SKI-606-treated cohorts as compared with the vehicle-treated group (Figure 1F, \( p=0.001 \)). These results
suggested that SKI-606 could significantly delay the progression of thyroid cancer and prevent lung metastasis in $Thrb^{Pv/Pv}Pten^{+/−}$ mice.

**TSH does not mediate inhibition of thyroid tumor growth by SKI-606 in $Thrb^{Pv/Pv}Pten^{+/−}$ mice**

Recent studies show that multi-targeted tyrosine kinase inhibitors, such as sunitinib and sorafenib, could influence thyroid functions (28, 29). It is known that TSH is a major stimulator of thyrocytes proliferation. To evaluate whether the decreased size of thyroid tumors after treatment with SKI-606 could possibly be due to decreased TSH levels, we compared serum TSH and total T4 levels (Figure 2). We found a small, but significant increase in the levels of serum TSH by SKI-606 treatment as compared with vehicle treatment (Figure 2A). A very small increase was also detected for total T4 (Figure 2B). These data suggested that the decreased thyroid tumor size in SKI-606-treated mice was not due to the effects of TSH.

**SKI-606 delays tumor progression by preventing de-differentiation and inhibiting cell proliferation in thyroids of $Thrb^{Pv/Pv}Pten^{+/−}$ mice**

To understand how SKI-606 inhibited thyroid tumor growth, we evaluated its effect on cell differentiation and proliferation. Remarkably, the follicular structure of thyroid gland and cellular polarity were largely maintained, similar to that of normal thyroid in the SKI-606-treated group as compared with vehicle-treated mice (Figure 3A). The cell population in thyroid was clearly decreased, and the quantification of cell density showed a 38% decrease after SKI-606 treatment as compared with vehicle treatment (Figure 3C, $p=0.003$). The anti-proliferative effect of SKI-606 was clearly evident by the reduced staining of Ki-67 in the nucleus of thyrocytes of $Thrb^{Pv/Pv}Pten^{+/−}$ mice (Figure 3B- panel b) as compared with the control (Figure 3B- panel a). Cells
positively stained with Ki-67 were scored among total cells examined. Figure 3D shows ~50% reduction in the positively Ki-67 stained cells, indicating treatment of SKI-606 led to inhibition of cell proliferation.

We further studied the protein abundance and activity of key regulators involved in cell cycle progression in thyroid tumors affected by SKI-606 treatment. Western blot analysis shows that the protein levels of cyclin B1, cyclin E, and cyclin D1 were reduced by 20-50% after SKI-606 treatment (Figure 4Aa, Ab, Ac). The levels of cyclin-dependent kinases (CDK4 and CDK6) were also lower in the SKI-606-treated group than the vehicle-treated group (Figure 4Ad, Ae). In addition, SKI-606 treatment decreased the phosphorylation of the retinoblastoma (Rb)(S807/811 and S780) (Figure 4Ba, Bb). In its unphosphorylated state, Rb acts as a negative regulator of cell cycle progression by binding to and inhibiting critical regulatory proteins, including members of the E2F family of transcription factors (Figure 4B). The intensities of the bands in Figure 4A and 4B were determined and the quantitative comparison was shown in Figure 4C. These results suggest that inhibition of Src signaling pathway by SKI-606 reduced thyroid tumor growth by inhibiting cell proliferation and cell cycle progression.

We also evaluated the effect of SKI-606 on apoptotic pathways. That inhibition of Src activity by SKI-606 did not alter cell apoptosis in $Thrb^{pv/pv} Pten^{+/−}$ thyroids was indicated by the absence of changes in the protein levels of the proapoptotic factors Bad, Bax, and Bcl-XL (Figure 4D). No changes of cleaved caspase-3 or cleaved poly ADP ribose polymerase (PARP) were detected by Western blot analysis after treatment with SKI-606 (data not shown).

**Treatment with SKI-606 inhibits the activation of MAPK pathways**

MAPKs including extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNKs), and p38 MAPK isoforms are serine/threonine-specific kinases...
that respond to extracellular stimuli and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival (30). Src has an important role in the receptor tyrosine kinase-mediated proliferative responses through the activation of the Ras-Raf-MEK-MAPK signaling pathways (31). We therefore evaluated the levels of phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated p38 isoforms (p-p38) by Western blotting. Figure 5A shows that after treatment of SKI-606, the levels of p-ERK1/2 (Figure 5Aa) and p-p38 MAPK (Figure 5Ac) were significantly lower in SKI-606-treated than in vehicle-treated thyroids of $\text{Thrb}^{PV/PV}\text{Pten}^{**}$ mice. The phosphorylated JNK (p-JNK) level, which plays an important role in inflammatory response and cancer microenvironment, was also lower in SKI-606 treated mice than in control groups (Figure 5B). The intensities of the bands in Figure 5A and 5B were determined and the quantitative comparison was shown in Figure 5C. The ratios of p-ERK/total ERK, p-p38/total p38 and p-JNK/total JNK was decreased 95%, 80% and 65%, respectively, indicative of marked attenuation of MAPK-ERK signaling in mice treated with SKI-606. These data indicate that inhibition of Src by SKI-606 led to blocking of downstream signaling via inhibition of phosphorylation cascades to decrease thyroid tumor growth and increase survival.

**Src inhibition by SKI-606 reduces the epithelial-mesenchymal transition in thyroid carcinogenesis**

The migration of tumor cells through matrix barriers, tissue compartments, vessels, and organ boundaries is essential in the development of distant metastasis (8). Previously, Src was shown to promote the expression of matrix-degrading proteases such as metalloproteinases (MMPs) by diverse mechanisms. Via the ERK signaling pathway, the expression of MMP-9 is elevated, and via the CAS-Rac-JNK and paxillin-Rac-JNK pathways, the expression of MMP-2 and MMP-9 is up-regulated (29, 32, 33).
We therefore evaluated the expression levels of MMP-9 and MMP-2 after treatment of $\text{Thrb}^{Pv/Pv \text{Pten}^{+/\}}$ mice with SKI-606. Consistent with the decreased activation of ERK and JNK signaling pathways (see Figure 5B), the protein abundance of MMP-9 was decreased 90% (Figure 6Aa and bars 1 & 2 of Figure 6B). Figure 6Ab shows that while pro-MMP-2 protein levels were similar between vehicle- and SKI-606-treated $\text{Thrb}^{Pv/Pv \text{Pten}^{+/\}}$ mice (bars 3 & 4, Figure 6B), the active-MMP-2 protein levels were decreased 50% in SKI-606-treated $\text{Thrb}^{Pv/Pv \text{Pten}^{+/\}}$ mice (bars 5 & 6, Figure 6B). That SKI-606 affected the conversion of pro-MMP-2 to active MMP-2 was indicated by the decreased ratios of active-MMP-2/pro-MMP-2 (from 2.34 to 0.96 for vehicle- and SKI-606-treated mice, respectively). Moreover, as shown in bars 7 & 8, the total MMP-2 protein levels were decreased 50% in SKI-606-treated $\text{Thrb}^{Pv/Pv \text{Pten}^{+/\}}$ mice. We further determined whether the expression of MMP-9 and MMP-2 was also affected by SKI-606 at the mRNA levels by real-time quantitative PCR. Figure 6C shows that SKI-606 treatment decreased the mRNA expression of MMP-9 and MMP-2 (90% and 80%, respectively). Taken together, these data indicate that SKI-606 decreased the expression of MMP-9 and MMP-2 both at the mRNA and protein levels. Moreover, the conversion of pro-MMP-2 to the active form was also reduced by SKI-606 treatment. Active MMP-2 was decreased 50% without significant change in pro-MMP-2 protein levels (Figure 6Ab and 6B). Real-time quantitative PCR data also indicated that mRNA expression of both MMP-9 and MMP-2 were also decreased by SKI-606 (Figure 6C, $p=0.04$, and $p=0.009$, respectively). The reduction of these proteases led to reduction in tumor cell migration.

We also examined the markers of EMT after treatment of $\text{Thrb}^{Pv/Pv \text{Pten}^{+/\}}$ mice with SKI-606. E-cadherin is involved in cell-cell adhesion, and activation of Src is known to decrease E-cadherin expression. As shown in Figure 6Da, treatment with SKI-606 led to 2.2-fold increases of E-cadherin protein abundance as compared with vehicle-treated
thyroids (see also the quantification in Figure 6E). Other EMT markers, vimentin and slug (snail 2), involved in invasion and tumor progression in the mesenchymal phenotype were decreased after treatment with SKI-606 (Figure 6Db and Dc, respectively). Taken together, our data indicate that reduction of Src signaling by SKI-606 blocks cancer progression and lung metastasis at least, in part, via inhibition of EMT in thyroid cancer.
Discussion

Genetic mutations of Src are very rare. But aberrantly activated Src and its downstream effector, FAK, are frequent in human cancers. Over-activation of Src increases cell growth and survival as well as promotes the reorganization of the cytoskeleton and decreases cell-cell and cell-matrix adhesion. These activities of Src ultimately facilitate cell motility and invasion (8). Thus, inhibition of Src is a promising target for controlling primary tumor growth, invasion, and metastasis. In thyroid cancer, the increased expression of Src and FAK has been reported, and over-expressed FAK has been proposed to be a marker of invasive potential in thyroid cancer (10-12). These findings suggested that Src could be a potential molecular target for treatment of thyroid cancer. The availability of Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mouse model that exhibits aggressive tumor progression and metastasis (17) provided us with an opportunity to test this possibility \textit{in vivo}. In the present study, we showed that Src-FAK signaling was over-activated (see Figure 1A and B) and therefore suitable for the evaluation of the effects of inhibiting this pathway on thyroid carcinogenesis. We showed that when the p-Src was inhibited by SKI-606, the survival of Thrb\(^{PV/PV}\)Pten\(^{+/−}\) was prolonged, the thyroid growth was markedly decreased, the occurrence of tumor invasion and metastasis was delayed and the degree was less severe. These findings clearly show that activation of Src-FAK signaling is critical in thyroid carcinogenesis. More importantly, the present study has identified an important potential molecular target for treatment of thyroid cancer.

By the use of SKI-606 in inhibiting the Src-FAK signaling, the present studies have uncovered downstream effectors critical in thyroid carcinogenesis of Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice. The key cell cycle regulators such as cyclins (D1, B1 and E), CDKs (CDK4 and 6), Rb and E2F1 were downstream effectors in mediating the proliferation of thyroid tumor cells of Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice. Moreover, in addition to p38 MAPK, shown previously a critical downstream effector of Src-FAK signaling in the
carcinogenesis of *Thrb<sup>Pv/PV</sup> mice* (22), ERK and JNK pathways were also found to participate in the carcinogenesis of *Thrb<sup>Pv/PV Pten<sup>+/−</sup></sup> mice*. These findings suggest that *Pten* haploid deficiency not only exasperates the PI3K-AKT signaling, but also expands the pathways downstream of Src-FAK signaling to contribute to thyroid carcinogenesis of *Thrb<sup>Pv/PV Pten<sup>+/−</sup></sup> mice*. The identification of these downstream effectors of Src-FAK signaling raises the possibility that intervention of phosphorylation cascades by kinase inhibitors in the ERK and JNK pathways would offer additional benefits in the treatment of thyroid cancer. Currently, MEK inhibitors, AZD6244 (NCT00970359) and GSK1120212 (NCT01438554), leading to blocking the activities of immediate downstream effector, ERK, are in clinical trials of refractory thyroid cancer. JNK inhibitors, such as SP600125 and AS601245, are in preclinical stage. Thus, the present preclinical findings would be useful in the consideration of using SKI-606 as a single agent upstream inhibitor of Src-FAK-ERK/JNK signaling or in combination with the inhibitors of ERK and JNK for effective treatment of thyroid cancer patients.

It is known that TSH is a major stimulator of thyrocyte proliferation. However, it is important to note that the reduced thyroid growth observed in mice treated with SKI-606 did not involved TSH. A small, but significant increase in the TSH and T4 levels were detected in *Thrb<sup>Pv/PV Pten<sup>+/−</sup></sup> mice* treated with SKI606, arguing against the role of TSH in the reduction of thyroid growth by SKI-606. These observations suggest that activation of Src-FAK signaling most likely largely responsible for propelling proliferation of thyroid tumor cells of *Thrb<sup>Pv/PV Pten<sup>+/−</sup></sup> mice*.

It is currently unclear whether the effect of SKI-606 on thyroid carcinogenesis of *Thrb<sup>Pv/PV Pten<sup>+/−</sup></sup> mice* can also act via the inhibition of Abl kinase. While it was reported that in chronic myeloid leukemia, SKI-606 could inhibit the aberrantly activated Abl kinase activity in Abl-Bcr fusion protein (34), yet it is unknown whether Abl could be
activated via Abl-Bcr re-arrangements in thyroid cancer and thyroid cancer cell lines. This question awaits further investigation.

The present studies also revealed that Src inhibition by SKI-606 reduced the extent of de-differentiation of thyroid tumor cells during tumor progression as evidenced by regaining more normal thyroid follicular structures. Microenvironmental changes in cell-cell and cell-matrix adhesion and the activation of EMT play an important role in cancer cell invasion and metastasis. We found that E-cadherin, essential for establishing cell polarity and maintaining epithelial integrity and cellular differentiation (35) was increased after treatment with SKI-606. Consistent with our observations, decreased expression of E-cadherin was reported to associate with de-differentiation, increasing frequencies of distant metastasis and poor prognosis of follicular thyroid cancer (36, 37). Moreover, we also found that markers of the mesenchymal invasive phenotype, vimentin and slug, were down-regulated by Src inhibition. While detailed molecular pathways leading to decreased de-differentiation of tumor cells by SKI-606 treatment is not clear, it is reasonable to postulate that such events could be mediated by the changes in MAPK signaling observed in the present study. This notion is consistent with the previous reports in which inhibition of the MAPK and TGFβ signaling pathways was shown to decrease EMT and thyroid cancer cell de-differentiation (38). That treatment of SKI-606 could lead to inhibition of de-differentiation of thyroid tumor cells could offer additional benefits to thyroid cancer patients with the possibility of increasing efficacy of radioiodine therapy and more positive prognosis. In summary, the present study clearly shows that over-activation of Src-FAK signaling promotes thyroid carcinogenesis and that Src and its several other downstream effectors are potential molecular targets for treatment of thyroid cancer.
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References


Figure 1. The effects of SKI-606 on survival, tumor growth, vascular invasion, and lung metastasis of thyroid cancer in \(Thrb^{Pv/P}\)Pten\(^{+/−}\) mice. (A) Src signaling pathway is activated in the thyroids of \(Thrb^{Pv/P}\)Pten\(^{+/−}\) mice as comparing with wild-type mice (n=3 for each group). Western blot analysis of phospho-Src (p-Src), total Src, phospho-focal adhesion kinase (p-FAK), total FAK, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. (B) SKI-606 effectively inhibited activation of Src and FAK in \(Thrb^{Pv/P}\)Pten\(^{+/−}\) mice (n=4) as compared with wild-type mice (n=4). Western blot analysis of p-Src, total Src, p-FAK, total FAK, and GAPDH as a loading control. (C) Survival curves for \(Thrb^{Pv/P}\)Pten\(^{+/−}\) mice treated with SKI-606 (n=11; 150 mg/kg body wt) or vehicle (n=15) orally 5 times a week from 6 weeks of age until they had to be euthanized due to sickness. Data presented by Kaplan-Meier methods and analyzed by log-rank test. (D) Thyroids of vehicle-treated or SKI-606-treated \(Thrb^{Pv/P}\)Pten\(^{+/−}\) mice were dissected from moribund mice at the age of 2.8 -5 months and 3.4 -6.6 months, respectively. The data are presented as ratios of thyroid weight to total body weight. The data are presented by median and analyzed by Mann-Whitney U test. (E) The development of vascular invasion in \(Thrb^{Pv/P}\)Pten\(^{+/−}\) mice after treatment with SKI-606 (n=11) or vehicle only (n=15). The data are presented by Kaplan-Meier methods and compared by log-rank test. (F) The development of lung metastasis in \(Thrb^{Pv/P}\)Pten\(^{+/−}\) mice after treatment with SKI-606 (n=11) or vehicle only (n=15). The data are presented by Kaplan-Meier methods and compared by log-rank test.

Figure 2. Serum TSH and total T4 levels after administration of SKI-606 in \(Thrb^{Pv/P}\)Pten\(^{+/−}\) mice. Levels of serum TSH (A) and total T4 (B) in \(Thrb^{Pv/P}\)Pten\(^{+/−}\) mice treated with SKI-606 (n=11) or vehicle only (n=12) were determined as described in Materials and Methods. The data are presented by mean and analyzed by Student’s \(t\)-test.
Figure 3. SKI-606 reduces tumor growth by prevention of de-differentiation and inhibition of cell proliferation and in ThrβPV/PVpTεn+/− mice. (A) Representative microphotographs of hematoxylin-eosin staining on thyroid sections of SKI-606- (n=11) or vehicle-treated ThrβPV/PVpTεn+/− mice (n=15). a,c,e=vehicle-treated group; b,d,f=SKI-606-treated group. (B) Representative microphotographs of Ki-67 immunohistochemistry on thyroid sections of SKI-606- (n=3) or vehicle-treated ThrβPV/PVpTεn+/− mice (n=3). a=vehicle-treated group, b=SKI-606-treated group. (C) Comparison of relative cell density determined by cell counting in the two groups. (D) Thyroid cell proliferative index, determined by Ki-67 immunohistochemistry in the two groups.

Figure 4. SKI-606 reduces cell proliferation by inhibition of cell cycle progression but does not induce apoptotic pathways in ThrβPV/PVpTεn+/− mice. (A) Western blot analysis of cyclin B1, cyclin E, cyclin D1, cyclin dependent kinase (CDK) 4, CDK6, and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as a loading control after treatment with vehicle (n=3) or SKI-606 (n=3). (B) Western blot analysis of phosphorylated retinoblastoma (Rb) (S807/811, and S780), total Rb, E2F1, and GAPDH as a loading control after treatment with vehicle (n=3) or SKI-606 (n=3). (C) Quantification of relative expression of proteins associated with cell cycle progression in thyroid cancer after normalization by using GAPDH as loading control. (D) Western blot analysis of Bax, Bcl-XL, and GAPDH as a loading control after treatment with vehicle or SKI-606 in the thyroids of ThrβPV/PVpTεn+/− mice.
Figure 5. Treatment with SKI-606 inhibits mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinases (ERK1/2), p38 MAPK, and c-Jun N-terminal kinases (JNKs) in Thrβ<sup>PV/PV</sup>Pten<sup>+/−</sup> mice. (A) Western blot analysis of phosphorylated ERK1/2, total ERK1/2, phosphorylated p38 MAPK, total p38 MAPK, and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as a loading control after treatment with vehicle (n=6) or SKI-606 (n=4). (B) Western blot analysis of phosphorylated JNK, total JNK, and GAPDH as a loading control after treatment with vehicle (n=3) or SKI-606 (n=3). (C) Quantification of relative expression of phosphorylated proteins associated with Src-FAK and MAPK pathways in thyroid cancer after by using GAPDH as loading control.

Figure 6. Src inhibition by SKI-606 reduces the epithelial-mesenchymal transition (EMT) of thyroid cancer. (A) Western blot analysis of matrix metalloproteinase (MMP)-9, pro MMP-2, active MMP-2, and GAPDH as a loading control after treatment with vehicle (n=3) or SKI-606 (n=3). (B) Quantification of relative protein expression of MMP-9, pro MMP-2, active MMP-2 and total MMP-2. (C) Relative mRNA expression of MMP-9 and MMP-2 were determined by real-time PCR. Total RNA from thyroid tumors after treatment with vehicle (n=6) or SKI-606 (n=6) was prepared. Results are presented as fold change to the mRNA level of vehicle treated mice. (D) Western blot analysis of E-cadherin, vimentin, slug (snail-2), and GAPDH as a loading control after treatment with vehicle (n=6) or SKI-606 (n=4). (E) Quantification of relative expression of proteins associated with EMT in thyroid cancer after by using GAPDH as loading control.
Figure 4

A

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- Cyclin B1
- Cyclin E
- Cyclin D1
- CDK4
- CDK6
- GAPDH

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- pRb (S807/811)
- pRb (S780)
- Rb
- E2F1
- GAPDH

C

Relative band intensity

Vehicle

SKI-606

- Cyclin B1
- Cyclin E
- Cyclin D1
- CDK4
- CDK6 (pRb (S807/811))
- pRb (S780)
- Rb
- E2F1

D

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- Bad
- GAPDH
- Bax
- Bcl-XL
- GAPDH
Figure 5

(A) Western blot analysis showing the effects of SKI-606 on ERK and p38 MAPK phosphorylation. Panels a-e show the levels of p-ERK (T202/Y204), Total ERK (p42/44), p-p38 MAPK (T180/Y182), Total p38 MAPK, and GAPDH, respectively, in lanes 1 through 10.

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(B) Western blot analysis showing the effects of SKI-606 on JNK phosphorylation. Panels a-c show the levels of p-JNK (T183/Y185), Total JNK (p46/54), and GAPDH, respectively, in lanes 1 through 6.

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<th>Total JNK (p46/54)</th>
<th>GAPDH</th>
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(C) Bar graph showing the relative band intensity of p-ERK/ERK, p-p38/p38, p-JNK/JNK with vehicle and SKI-606 conditions.
Figure 6

A

KD
98

MMP-9

Pro-MMP-2

Active-MMP-2

Active/Pro MMP-2 ratio: 2.34 0.96

GAPDH

b

64

1 2 3 4 5 6

SKI-606 - +

B

Relative band intensity

Vehicle

SKI-606

1 2 3 4 5 6 7 8

MMP-9 Pro MMP-2 Active MMP-2 Total MMP-2

p=0.04 p=0.009

C

Relative mRNA expression

Vehicle

SKI-606

1 2 3 4

MMP-9 MMP-2

D

KD
98

E-cadherin

Vimentin

Slug

GAPDH

b

50

c

36

d

36

1 2 3 4 5 6 7 8 9 10

1 2 3 4 5 6

1 2 3 4

Vehicle

SKI-606

Relative band intensity

E-cadherin Vimentin Slug

1 2 3 4 5 6

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Won Gu Kim, Celine J. Guigon, Laura Fozzatti, et al.


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