Targeting FGFR/PDGFR/VEGFR impairs tumor growth, angiogenesis and metastasis by effects on tumor cells, endothelial cells and pericytes in pancreatic cancer

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Running title: Targeting FGFR/PDGFR/VEGFR in pancreatic cancer

Key words: FGFR, angiogenesis, pancreatic cancer, metastases

Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; FGF-1, fibroblast growth factor-1 (also named: acidic fibroblast growth factor); FGF-2, fibroblast growth factor-2 (also named: basic fibroblast growth factor); PDGF, platelet derived growth factor; VEGF, vascular endothelial growth factor; STAT3, signal transducer and activator of transcription 3; EGF, epidermal growth factor; Hsp90, heat-shock protein 90

Financial support: These studies were supported in part by the German Research Council (Deutsche Forschungsgemeinschaft (DFG) to S.A.L. and C.H.), the German Cancer Aid (Deutsche Krebshilfe, to O.S.), and grants from the University of Regensburg, Medical Faculty (ReForM-A and ReForM-C) (to S.A.L., C.M. and C.H.)

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ABSTRACT

Activation of receptor tyrosine kinases (RTKs) such as fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR) and vascular-endothelial growth factor receptor (VEGFR) have been implicated in tumor progression and metastasis in human pancreatic cancer. In this study we investigated the effects of TKI258, a tyrosine kinase inhibitor to FGFR, PDGFR and VEGFR on pancreatic cancer cell lines (HPAF-II, BxPC-3, MiaPaCa-2, L3.6pl), endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). Results showed that treatment with TKI258 impaired activation of signaling intermediates in pancreatic cancer cells, ECs and VSMCs, even upon stimulation with FGF-1, FGF-2, VEGF-A and PDGF-B. Furthermore, blockade of FGFR/PDGFR/VEGFR reduced survivin expression and improved activity of Gemcitabine in MiaPaCa2 pancreatic cancer cells. In addition, motility of cancer cells, ECs and VSMCs was reduced upon treatment with TKI258. In vivo, therapy with TKI258 led to dose-dependent inhibition of subcutaneous (HPAF-II) and orthotopic (L3.6pl) tumor growth. Immunohistochemical analysis revealed effects on tumor cell proliferation (BrdU) and tumor vascularisation (CD31). Moreover, lymph node metastases were significantly reduced in the orthotopic tumor model when treatment was initiated early with TKI258 (30 mg/kg/d). In established tumors, TKI258 (30 mg/kg/d) led to significant growth delay and improved survival in subcutaneous and orthotopic models, respectively. These data provide evidence that targeting FGFR/PDGF/VEGFR with TKI258 may be effective in human pancreatic cancer and warrants further clinical evaluation.
INTRODUCTION

Pancreatic cancer is the fourth leading cause for cancer-related death in the Western world (1). As conventional cancer treatments have little impact on disease course, almost all patients having pancreatic cancer develop metastases and die. To date, only surgical resection (possible in about 10–20% of patients) can increase the 5-year survival rate from 3% to 20%; systemic chemotherapy and/or radiation may only allow a marginal increase in survival (2, 3). Therefore, new therapies based on the molecular biology of pancreatic cancer are needed to improve overall survival of patients with this aggressive disease.

In human pancreatic cancer overexpression of receptor tyrosine kinases (RTKs) such as fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR) and vascular-endothelial growth factor receptor (VEGFR), as well as their corresponding ligands is a common event and is eventually associated with reduced patient survival (4-6). In particular, members of fibroblast growth factor (FGF) family have been shown to contribute to tumor cell proliferation and resistance to chemotherapy in human pancreatic cancer cell lines (7, 8). Furthermore, since the FGF family comprises 23 different ligands, other members such as FGF-7 have also been associated with an increase in tumor cell motility and enhancement of metastases (9). Therefore, targeting FGFR might improve current therapy concepts for pancreatic cancer patients.

However, expression of FGFR/PDGFR/VEGFR is not limited to cancer cells. In fact, these receptor systems are crucial mediators of tumor angiogenesis which is among the hallmarks of cancer (10, 11). With no doubt, vascular-endothelial growth factor-A (VEGF-A) is the major regulator in this complex process by direct effects on endothelial cells (ECs) (12). However, there is increasing evidence that crosstalk between VEGFs and FGFs plays an important role in the promotion of tumor angiogenesis by mutually regulating their expression (13-16). In addition, recruitment of pericytes (e.g. vascular smooth muscle cells, VSMC) by
PDGF-B is essential for maintenance of tumor angiogenesis, and cooperation of FGF-2 with PDGF-B has been shown to promote angiogenesis and metastasis formation in murine cancer models (17). Thus, targeting FGFR/PDGFR/VEGFR by a multiple receptor tyrosine kinase inhibitor (RTK) might be a promising approach for anti-angiogenic therapy concepts.

TKI258 (formerly CHIR258) (4-amino-5-fluor-3-[5-(4-methylpiperazin-1-yl)-1H-benzimidazol-2-yl]quinolin-2(1H)-one) is an orally available ATP-competitive inhibitor with activity against class III, IV and V RTKs, including FGFR, PDGFR and VEGFR (18). The substance has shown its anti-tumoral activity in various preclinical models, including mammary tumors, multiple myeloma, and colon cancer models (19-21). Due to its inhibitory effects on multiple tyrosine kinases, TKI258 is thought to act on tumor cells and endothelial cells as well as pericytes.

In the present study, we assessed the combined anti-angiogenic and antitumor activity of targeting FGFR/PDGFR/VEGFR with TKI258 in a pancreatic cancer model. In vitro, a substantial inhibition of motility and growth factor-induced signaling was observed. Furthermore, we evaluated its effects on tumor growth and metastasis in experimental murine models of subcutaneous and orthotopic pancreatic cancer in vivo. Results provide evidence that combined inhibition of FGFR/PDGFR/VEGFR might be a novel strategy to improve the outcome of patients with pancreatic cancer.
MATERIAL AND METHODS

Cell Culture and Reagents.

Human pancreatic cancer cell lines BxPC-3, MiaPaCa2 and HPAF-II were obtained from the American Type Culture Collection (ATCC) and L3.6pl cells were kindly provided by Dr. I. J. Fidler (The University of Texas M.D. Anderson Cancer Center). Human umbilical vascular endothelial cells (HUVEC, endothelial cells, ECs) and vascular smooth muscle cells (VSMC) were purchased from Promocell. No further authentication was done for tumor cell lines. Tumor cells were cultured in DMEM (PAA Laboratories) supplement with 15% FCS and maintained in 5% CO₂ at 37°C as described (22). Recombinant human VEGF-A, PDGF-B, FGF-1, FGF-2 and FGF-7 were purchased from R&D Systems (Wiesbaden, Germany). The FGFR/PDGFR/VEGFR inhibitor TKI258 was kindly provided by Novartis Oncology (Basel, Switzerland) and dissolved in DMSO (in vitro) and water (in vivo), respectively (Fig. 1A).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assays.

To evaluate cytotoxic effects of NVP-TKI258 on tumor cells, HUVEC and VSMC, cells were seeded in 96-well plates (1×10³ per well) and exposed to various concentrations of TKI258. Respective concentrations of DMSO were added to controls. We used the MTT assay to assess cell viability as described before (22).

Migration Assays.

To determine the effect of TKI258 treatment on cell motility, migration assays were performed using modified Boyden chambers as previously described (22, 23). Briefly, 5 × 10⁴ tumor cells were resuspended in 1% FCS-DMEM and seeded into inserts with 8µm pores (Becton Dickinson Bioscience, Heidelberg, Germany). In tumor cells, FGF-1 (50 ng/ml) and FGF-7 (50 ng/ml) were used as chemoattractant. After 24 and 48 hours, cells were fixed and migrated cells stained (Diff-Quick reagent; Dade Behring, Newark, NJ). Cells that migrated
through the filters were counted in four random fields and average numbers were calculated. In VSMCs, PDGF-B (10 ng/ml) and FGF-2 (50 ng/ml) were used as chemoattractant whereas motility in ECs was assessed with FGF-2 (50 ng/ml) and VEGF-A (50 ng/ml).

**Western Blot Analyses for Activated Signaling Pathways and Receptor Expression.**

Experiments were performed at a cellular density of 60% to 70%. Unless otherwise indicated, cells were incubated with TKI258 (0.5 µmol/l) before stimulation with either VEGF-A (10 ng/ml), PDGF-B (10 ng/ml), FGF-1 (50 ng/ml) or FGF-2 (50 ng/ml). Whole-cell lysates were prepared as described elsewhere (24). Protein samples (40 µg) were subjected to Western blotting on a denaturing 10% SDS-PAGE. Membranes were sequentially probed to indicated signaling intermediates with antibodies against phospho-AktSer473, Akt, phospho-ERKThr202/Tyr204, ERK, phospho-STAT3Tyr705, STAT3, N-Cadherin, E-Cadherin, survivin, phospho-c-RafSer289/296/301, phospho-c-RafSer338, c-Raf, phospho-FAKTyr925, FAK, FGFR3, EGF-R, Hsp90 (Cell Signaling Technologies, Beverly, MA), and phospho-VEGFR2, VEGFR2, PDGFRα, PDGFRβ and β-actin (Santa Cruz Biotechnologies, Santa Cruz, CA). Antibodies were detected by enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ).

**Real-time PCR Analysis.**

Expression of FGFRs (FGFR1–3) and the effects of TKI258 on the expression of DLL4, survivin, FGF-2, EGF, VEGF-A, PDGF-B, Tie2 were determined by real-time PCR from cell culture and tumor tissue. For this purpose, total RNA was isolated using Trizol reagent (Invitrogen) and subsequently purified by ethanol precipitation. For each RNA sample, a 1 µg aliquot was reversely transcribed into cDNA using the Superscript II Kit (Quiagen). Selected primer pairs for PCR were as follows: DLL4 (5’-TGCAGGAGTTCATCAACGAG and 3’-GAAATTGAAAGGCAGGTTGGA), survivin (5’-GGACCACCGCATCTCTACAT and 3’-GACAGAAAGGAAAGCGCAAC), FGFR1 (5’-CAACCGTGTGACCAAAGTGG and
3´TCCGACAGGTCTTCTTCCG), FGFR2IIIb (5´-AAGGTTTACAGCGATGCCCA and 3´-AGGAGCGCAGACTTCTGCATT), FGFR2IIIC (5´-GTGTTAACACCACGGGACA and 3´-TGGCAGAACTGTCAACCATG), FGFR3 (5´-GTGGCTGGAGCTACTTCC and 3´-ATCCTTAGCCCAAGACCAGTGG), FGFR-2 (5´-AGCGGCTGTACTGCAAAAAC and 3´-TTCTGCTTTGAAGTTGACTGTGTGAT), EGF (5´-AAGAATGCGGGGTCAAC and 3´-TGAAGGTGGTTGCTTAGGTGACC), Tie2 (5´-GAAGCCGCCCTGAAGTGATG and 3´-CTTGGCAATGGTGGTACTCTCT), VEGF-A (5´-GCAGCTGGAGTAAAGCAACG and 3´-GGTTCCGGAAACCCTGAG), PDGF-B (5´-TGGTGAGTTGAAAGCTCATCTC and 3´-GTCTTGCACTCGGCGATTA) and GAPDH (5´-GCAGGGCTCCAGACATCAT and 3´-CCAGCCCAGCGTCAAAGGTG). Primers were optimized for MgCl2 and annealing, and PCR products were confirmed by gel electrophoresis. RT-PCR was done using the LightCycler system and Roche Fast-Start Light Cycler-Master Hybridization Probes master mix (Roche Diagnostics).

Animal Models.

Eight-week-old male athymic nude mice (BALB/c nu/nu) (Charles River, Germany) were used for experiments, as approved by the Institutional Animal Care and Use Committee of the University of Regensburg and the regional authorities. In addition, experiments were conducted according to “Guidelines for the Welfare of Animals in Experimental Neoplasia” published by The United Kingdom Coordinating Committee on Cancer Research. Effects of FGFR/PDGFR/VEGFR inhibition with TKI258 were first evaluated in an orthotopic pancreatic cancer model using metastatic L3.6pl cancer cells. Briefly, 1 × 10^6 human pancreatic cancer cells were injected into the pancreatic tail of mice. Mice were randomized into 3 groups (n = 9–10 per group) receiving either vehicle (controls) or TKI258 (15 mg/kg/d or 30 mg/kg/d) by oral gavage. Treatment started 7 days after tumor cell inoculation. After 31 days, mice were sacrificed, tumors excised and weighed and incidence of liver and lymph
node metastases was determined. Furthermore, tumors were either paraffin-embedded or OCT-embedded for immunohistochemical analyses. Effects of TKI258 on established orthotopic tumors were evaluated similarly (n=8/group). In this model treatment (30 mg/kg/d) was initiated when tumors were palpable on day 15. Mice were individually assessed every day and sacrificed when signs of progressive tumor disease were observed. Tumors were excised, weighed and prepared for Western blot analyses and PCR. PCR was performed with pooled tissue samples from each group. Western Blot analyses on tumor tissues were performed as described elsewhere (22). Subsequently, the effects of FGFR/PDGFR/VEGFR inhibition with TKI258 on the growth of human pancreatic cancer cells (HPAF-II, L3.6pl) were investigated in subcutaneous tumor models. Cancer cells (1 × 10^6) were injected into the subcutis (right flank) of nude mice. In the first experiment with HPAF-II cells, mice were randomized into 2 groups (n = 7/group) receiving either vehicle (controls) or TKI258 (30 mg/kg, 3x/wk) by oral gavage. Treatment was started when tumors became palpable and was terminated on day 28. Within the second experiment, tumors (L3.6pl) were grown up to a size of 400 mm^3, and subsequently treatment with either vehicle or TKI258 (30 mg/kg/day) for 7 days was initiated. Tumor diameters were measured every day and tumor volumes calculated (width^2 × length × 0.5). When the experiment was terminated, subcutaneous tumors were excised and weighed. For immunohistochemical analyses, tumors were either paraffin-embedded or OCT-embedded.

**Immunohistochemical Analysis of Tumor Vascularisation and Tumor Cell Proliferation.**

Multiple cryosections were obtained from tumors for all immunohistochemical analyses. CD31^+ vessel area was assessed using rat anti-mouse CD31/PECAM-1 antibody (Pharmingen San Diego) and peroxidase-conjugated goat anti-rat IgG (Jackson Research Laboratories, West Grove, PA) as previously described (24, 25). Antibody binding was visualized using stable diaminobenzidine. Images were obtained in four different quadrants of each tumor.
section (2 mm inside the tumor-normal tissue interface) at 40x magnification. Measurement of vessel area of CD31-stained vessels was done by converting images to grayscale and setting a consistent threshold for all slides using ImageJ software (version 1.33; NIH). Vessel areas were expressed as pixels per high-power field (22). To determine the amount of proliferating tumor cells, mice received intraperitoneal injections of BrdU (Sigma Aldrich, Germany; 1 mg/mouse) two hours prior to termination of animal studies. A commercially available BrdU detection kit (Becton Dickinson) was used to visualize BrdU-uptake of cells in sections of tumors. Briefly, sections were incubated with anti-BrdU antibody solution, followed by streptavidin-conjugated HRP-linked goat anti-mouse IgG2. Antibody binding was visualized by incubating slides in diaminobenzidine with the aid of hematoxylin counterstaining. BrdU-positive tumor cells were counted in four fields per tumor section at 20x magnification and averages were calculated (24, 25).

**Statistical Analysis.**

Statistical analyses were done using SigmaStat (Version 3.0). Results of *in vivo* experiments were analyzed for significant outliers using the Grubb’s test (www.graphpad.com). Tumor-associated variables of *in vivo* experiments were tested for statistical significance using the Mann-Whitney U test for nonparametric data or ANOVA followed by Tukey’s multiple comparison test for more than 2 groups. Effects on survival were determined by Kaplan-Meier analyses. The two-sided Student’s t test was applied for analysis of *in vitro* data. All results are expressed as the mean ± standard error of the mean (SEM).
RESULTS

_Treatment with TKI258 inhibits FGF signaling in tumor cells._

To determine the effects of TKI258 on pancreatic cancer cell lines (BxPC-3, MiaPaCa2, HPAF-II and L3.6pl), MTT assays were performed for up to 72 hours. Results showed no effects on growth of cancer cells within the first 48 hours for concentrations up to 5 µM. Incubation for longer time-points reduced MTT signals starting at a concentration of 2 µM (data not shown). Hence, for further _in vitro_ experiments concentrations between 0.5 and 1 µM were used.

First, the expression pattern of FGFRs (FGFR1–3) in pancreatic cancer cell lines was determined by RT-PCR and Western Blot and results showed that all used cell lines express these receptors (data not shown). Since FGF-1 is an important mediator for tumor cell proliferation and resistance against chemotherapy in pancreatic cancer, we evaluated the impact of TKI258 on FGF-1-induced signaling in pancreatic cancer cell lines. Pretreatment of cancer cells with TKI258 for 2 and 20 hours impaired constitutive and FGF-1-induced activation of ERK (Fig 1B). Of note, no phosphorylation of Akt was found upon treatment with FGF-1 (data not shown). Surprisingly, although all pancreatic cancer cell lines express PDGFRβ, no activation of signaling intermediates was observed upon stimulation with PDGF-B (data not shown). To further address the issue of chemoresistance, combined treatment of MiaPaCa2 pancreatic cancer cells with TKI258 and Gemcitabine was tested. This particular cell line was selected since a certain resistance of MiaPaCa2 against Gemcitabine has been reported (26). In MTT assays, a significant growth inhibition upon combined incubation was detected after 48 and 72 hours compared to Gemcitabine treatment alone (Fig. 1C). Similar results were obtained by counting cells upon incubation with Gemcitabine, TKI258, or the combination (data not shown). Furthermore, a dose-dependent decrease in survivin expression was found in pancreatic cancer cells on an mRNA (data not shown) and
protein level (Fig. 1E). We conclude from these data that blocking FGFR with TKI258 might improve therapy with Gemcitabine in pancreatic cancer.

**Effect of TKI258 on cancer cell motility.**

Signaling induced by FGF-1 is known to promote metastatic spread of cancer cells (27). Hence, we evaluated the effect of targeting FGFR on tumor cell motility using Boyden Chamber assays. Results showed that TKI258 impairs constitutive tumor cell motility after 24 and 48 hours (data not shown). In addition, FGF-1-induced tumor cell migration was significantly blocked by TKI258 (Fig. 1D). Similar results were obtained for FGF-7-induction of tumor cell motility (data not shown). To further determine the impact of TKI258 on constitutive motility, N-Cadherin and E-Cadherin expression was determined in cancer cells. Interestingly, treatment with the inhibitor led to a dose-dependent down-regulation of N-Cadherin, whereas E-Cadherin tended to be up-regulated (Fig. 1E). Taken together, our results demonstrate that blockade of FGFR with TKI258 leads to significant reduction of cancer cell motility *in vitro*, which could play a part in potential anti-metastatic effects *in vivo*.

**TKI258 impairs activation of endothelial cells.**

Induction of angiogenesis is important for tumor growth and metastases formation. Within this complex process, activation and recruitment of endothelial cells and pericytes is known to be crucial (12). Since TKI258 not only inhibits FGFR, but also VEGFR, we next assessed its effects on endothelial cells. In MTT assays, treatment with the inhibitor only led to moderate growth-inhibitory effects of TKI258 on endothelial cells, similar to those on tumor cells, even upon stimulation with VEGF-A and FGF-2 (data not shown). However, Western blot analyses of endothelial cells upon stimulation with VEGF-A revealed an inhibition of VEGF-A-induced phosphorylation of FAK. Furthermore, constitutive phosphorylation of c-Raf\(^{\text{Ser289/296/301}}\) and ERK was diminished (Fig. 2A). In addition, FGF-2-induced ERK and c-Raf\(^{\text{Ser338}}\) phosphorylation was impaired (Fig. 2B). On a functional level, EC motility was
induced by stimulation with either VEGF-A or FGF-2. This induction can be significantly impaired by treatment with TKI258 (Fig. 2C left and Fig. 2C right). Moreover, since TKI258 exhibits effects on VEGFR/c-Raf and FGFR/c-Raf activation in endothelial cells, we sought to investigate whether this inhibitor might also modulate factors involved in resistance to anti-VEGF therapy and survival of ECs (28). Therefore, RT-PCR was applied to determine expression of DLL4 and survivin in endothelial cells and a robust (> 50%) down-regulation was detected for both (Fig. 2D left and Fig. 2D right). In summary, treatment of endothelial cells with TKI258 impairs growth factor signaling leading to cell motility and shows promise to reduce endothelial factors involved in resistance to anti-VEGF therapy.

**Impact of TKI258 on pericytes (vascular smooth muscle cells).**

In addition to endothelial cells, pericytes are essential for development of tumor angiogenesis (29). Therefore, we included investigation of TKI258’s effects on this cell type into the study. Human vascular smooth muscle cells (VSMCs) were used as a source of pericytes. Using MTT assays, only minor inhibition of growth was found upon treatment for 72 hours, even when cells were stimulated with PDGF-B and FGF-2 (data not shown). However, since both PDGF-B and FGF-2 are involved in VSMC recruitment, we next determined the effects of TKI258 on activation of signaling intermediates. Western blot analyses revealed an inhibition of PDGF-B-induced Akt and ERK phosphorylation, as well as a reduction in phosphorylated STAT3 (Fig. 3A). In contrast, stimulation with FGF-2 had no effect on activation of either ERK or Akt in VSMC (Fig. 3B). On a functional level, motility of VSMCs was significantly impaired upon treatment with TKI258, only when cells were stimulated with either PDGF-B or FGF-2 (Fig. 3C left and 3C right). In addition, expression of survivin mRNA was significantly reduced, similar to our findings in ECs (Fig. 3D left). Since pericytes are a major source of FGF-2 in tumors, we furthermore analyzed effects of targeting FGFR/PDGFR/VEGFR on FGF-2 mRNA expression in VSMCs. Results show a
reduction in FGF-2 mRNA levels upon treatment with TKI258 (Fig. 3D right). We conclude from these experiments that TKI258 elicits effects on PDGF-B and FGF-2-induced pericyte recruitment.

**Effects of FGFR/PDGFR/VEGFR inhibition on tumor growth.**

Our results so far indicate that treatment with a multityrosine kinase inhibitor to FGFR/PDGFR/VEGFR might have significant effects on tumor growth, angiogenesis and metastatic spread of pancreatic cancer. To further analyze this issue, an orthotopic pancreatic cancer model (L3.6pl) was used. Seven days after tumor cell implantation, mice were treated with two different doses of TKI258 (15 mg/kg/d and 30 mg/kg/d), or vehicle control. Upon termination of the experiment after 24 days of treatment (31 days after tumor cell implantation), a significant inhibition of tumor weight was found in the higher dosing group (Fig. 4A). Although the lower dosing group also showed a reduced tumor weight, this did not reach statistical significance. Moreover, a dose-dependent reduction in both lymph node and liver metastases was found upon treatment with TKI258 (Tab. 1). In the higher dosing group, reduction of lymph node metastases was significant, whereas reduction of liver metastases was noted, but not significant. To estimate the effects of our treatment on tumor vascularisation, CD31+ vessel area was determined. Again, treatment with 30 mg/kg/d showed significant reduction in tumor vascularisation, whereas lower dosing with 15 mg/kg/d demonstrated no effect (Fig. 4B). In contrast, tumor cell proliferation determined by BrdU staining was significantly reduced at both, 15 and 30 mg/kg/d (Fig. 4C). To further exclude that anti-tumoral effects are cell line specific, a subcutaneous tumor model was used with HPAF-II cells. Consistent with our results from the orthotopic model, a significant inhibition of tumor growth was observed by treatment with TKI258 (30 mg/kg/d) (Fig. 4D), which was also reflected by final tumor weight (data not shown). In summary, these experiments show a
clear dose-dependent inhibition of tumor growth and metastatic spread by blockade of FGFR/PDGFR/VEGFR.

**TKI258 delays growth of established tumors.**

Since most of pancreatic cancer patients have large tumors that are not resectable upon diagnosis, we further assessed the effects of TKI258 on established subcutaneous tumors (L3.6pl). When tumor size reached approximately 400 mm³, treatment was initiated with 30 mg/kg/d. Up to 4 days after initiation of treatment, no significant difference in tumor growth was observed. However, from a size of 600 mm³, TKI258-treated tumors stopped growing. After a total of 6 days of treatment, growth inhibition by TKI258 was significant compared to controls, although no tumor shrinkage in the treatment group was observed (Fig. 5A). When tumors were analyzed for proliferating tumor cells and tumor vascularisation, a significant reduction of BrdU positive cells and CD31⁺ vessel area was found (data not shown). These results were subsequently confirmed in an orthotopic tumor model. Control mice had to be sacrificed after a median of 23 days, whereas TKI258 treated mice showed a median survival of 40 days. Consistent with results from the subcutaneous model, therapy with TKI258 (30 mg/kg/d) showed a delay in tumor growth which led to a significant improvement of survival in TKI258-treated mice (P<0.05) (Fig. 5B). In treated mice, after a longer time of survival, tumors were still slightly smaller than in untreated animals, although this difference was not significant (control: 1.38g ± 0.31 vs. TKI258: 1.07 ± 0.23). In addition, a trend towards reduced metastasis was observed upon therapy with TKI258 (lymph nodes metastases: 6/8 controls vs. 3/8 in TKI258 group; liver metastases: 5/8 controls vs. 3/8 in TKI group). Again this did not reach statistical significance indicating only a sign of progressive disease. To further assess mechanisms of resistance development, Western blot and PCR were performed on tumor tissue. On a protein level, results showed that inhibition of FGFR/PDGFR/VEGFR led to down-regulation of several growth factor receptors such as VEGFR2, PDGFRβ,
FGFR3, EGFR, as well as reduced expression of Hsp90 and survivin in tumor tissue. In turn, TKI258-treated tumors showed an increase in PDGFRα expression (Fig. 5C). However, an up-regulation of Tie2 (2-fold) (Fig. 5D) and PDGF-B (3-fold) mRNA was observed, whereas VEGF-A, FGF2 and EGF were slightly reduced upon FGFR/PDGFR/VEGFR inhibition (data not shown). Hence, our results clearly demonstrate that targeting FGFR/PDGFR/VEGFR has the potential to impair tumor growth even in established tumors.
DISCUSSION

Human pancreatic cancer remains associated with a high mortality since most patients present in advanced stages of the disease. Therefore, novel approaches are needed to improve the prognosis for these patients. Within our current study, we assessed the effects of targeting FGFR/PDGFR/VEGFR by using a novel multityrosine kinase inhibitor (TKI258) in pancreatic cancer models. Our results show that inhibition of these RTKs impairs multiple signaling pathways in tumor cells, endothelial cells and pericytes. Furthermore, we found a dose-dependent inhibition of pancreatic cancer growth and metastasis formation in vivo. In addition, survival of mice with established orthotopic tumors was significantly improved. Taken together, our results suggest that the combination of targeting FGFR, PDGFR and VEGFR might be a novel way to improve outcome of patients with pancreatic cancer.

Previous studies have shown the anti-tumoral activity of targeting multiple RTKs with TKI258 in a variety of tumor models (20, 21, 30). However, these studies focused on the inhibitory capacity of TKI258 on activation of PDGFRβ and VEGFR2 (21, 30). In contrast, a recent report by Dey and coworkers describes that inhibition of FGFR in a breast cancer model is a crucial step for TKI258’s antitumoral activity (20). In our model we confirmed the effects of TKI258 on FGF-1-induced phosphorylation of ERK in tumor cells. Moreover, FGF-1-induced motility of pancreatic cancer cells was significantly suppressed by FGFR blockade with TKI258. However, in contrast to Dey and coworkers, we found no phosphorylation of Akt upon stimulation with FGF-1, which might be due to the fact that pancreatic cancer cells rely on other mechanisms of Akt phosphorylation than breast cancer cells (20). We can add from our experiments that a decrease in survivin expression may be a factor. This is of particular importance since survivin is expressed in pancreatic adenocarcinoma and has been associated with resistance to chemo- and radiotherapy (31, 32). This observation is further underlined by the improvement of chemosensitivity to Gemcitabine in MiaPaCa2 cells.
Therefore, our data indicate that targeting FGFR with TKI258 in pancreatic cancer cells reduces invasive properties and improves sensitivity to Gemcitabine in pancreatic cancer cells.

FGF signaling is not only involved in motility and survival of tumor cells, it is a crucial component of tumor angiogenesis (10, 12). In particular, VEGF-A and FGF-2 are known to be major mediators of endothelial cell recruitment in terms of proliferation, migration and survival (33). Alireza and coworkers have described a mechanism of chemoresistance in endothelial cells mediated via FGF-2-induced activation of Raf-1 (34), prompting our investigation into the effects of TKI258 on VEGF-A- and FGF-2-induced signaling. We found an inhibition of multiple signaling pathways including ERK, FAK and c-Raf. On a functional level, TKI258 was able to reduce both VEGF-A and FGF-2-induced endothelial cell migration. Moreover, upon treatment with this multikinase RTK inhibitor, a significant reduction in DLL4 and survivin expression was detectable. This is of particular interest since DLL4 has been associated with resistance to anti-VEGF therapy in some tumor models (28, 35, 36). In addition, pericytes (or VSMCs as representatives of pericytes) are essential components of tumor angiogenesis. In particular, Nissen and coworkers described a synergistic prometastatic and proangiogenic activity of PDGF-B and FGF2 (15). Using TKI258 we found that activation of signaling intermediates Akt and ERK by PDGF-B was diminished. Furthermore, PDGF-B- and FGF2 -mediated motility of VSMCs was impaired. In view of these data, one might speculate that targeting FGFR, PDGFR and VEGFR by TKI258 might improve current anti-angiogenic therapy strategies in solid tumors.

We used an orthotopic tumor model to further evaluate our findings. The advantage of this model is the high metastatic potential combined with an appropriate microenvironment. As expected from our *in vitro* data, treatment with TKI258 led to a significant reduction in tumor growth in a dose-dependent manner. One might speculate that the improved inhibition at the
higher dosing group (30 mg/kg/d) might be mediated via anti-angiogenic effects since CD31+ vessel area was only reduced in this group. However, the effective dose of TKI258 30 mg/kg/d is in the lower range compared to that described in former reports (20, 21, 30, 37). In this model we also found a significant inhibition of metastasis formation in the lymph nodes and a trend to fewer liver metastases. These results are consistent with our in vitro data showing inhibition of tumor cell motility. However, several studies before have evaluated the effects of PDGFR and/or VEGFR inhibition on metastasis in pancreatic cancer models (23, 38-40). Although a strong inhibition of metastasis has been described by most of them, clinical studies did not confirm these findings (41-43). Nonetheless, in addition to targeting PDGFR/VEGFR, TKI258 elicits strong effects on FGFR which adds a novel important anti-tumoral effect to our potential treatment arsenal.

Since most patients present in an advanced clinical stage of disease, we assessed effects of combined inhibition of FGFR/PDGFR/VEGFR on established tumors. First, treatment with 30 mg/kg/d led to a significant inhibition of subcutaneous tumor growth after 6 days of treatment. The reduced tumor cell proliferation and decreased vessel area observed in these established tumors support the idea of targeting FGFR/PDGFR/VEGFR in advanced disease states. These results were further confirmed for the first time in established orthotopic pancreatic tumors where TKI258 (30 mg/kg/d) significantly improved survival of mice. Although growth inhibition in established tumors upon TKI258 treatment has been reported in a model of colon cancer, 2- to 4-fold higher doses of the inhibitor were used in the model (21). It is, therefore, possible that inhibition of angiogenesis and tumor cell proliferation can be achieved far below the maximum tolerated dose, which is of particular interest since combination with conventional chemotherapy might improve these results. Furthermore, since TKI258 is effective against tumor angiogenesis, we sought to evaluate mechanisms of resistance upon tumor progression in the orthotopic model. Recently, Cascone showed that
increased activity of EGFR and FGFR pathways is associated with resistance against VEGF inhibitors (44). Surprisingly, we detected a down-regulation of FGFR3 and EGFR expression in our experiments. One might speculate that this is based on the fact that TKI258 blocks FGFR and PDGFR in addition to VEGFR. In contrast, an up-regulation of PDGFRα in TKI258-treated tumors was detected which is of particular interest since PDGFRα is associated with tumor progression and metastasis in various cancer entities (45, 46). Moreover, PDGFRα is the main receptor for PDGF-C which in-turn has been reported to be up-regulated in tumor-associated fibroblasts that mediate resistance to anti-VEGF therapy (47). However, as mentioned above, dosing for TKI258 was chosen far below the maximum tolerated dose in our experiments which offers the possibility to combine the inhibitor with conventional chemotherapy to further improve effects on survival. Nevertheless, taken together, our results clearly indicate that inhibition of multiple RTKs by TKI258 has the potential to impair tumor growth even in advanced stages.

In conclusion, the present study shows that targeting FGFR/PDGFR/VEGFR may lead to an effective inhibition of tumor growth and metastasis in pancreatic cancer even in advanced tumor stages. Inhibition of signaling in tumor cells combined with antiangiogenic effects on endothelial cells by disruption of FGF signaling and inhibition of DLL4 expression may improve current antineoplastic therapy strategies for the treatment of pancreatic cancer patients. These results provide evidence for the use of FGFR/PDGFR/VEGFR multityrosine kinase inhibitors in clinical trials for pancreatic cancer.
ACKNOWLEDGEMENTS

The authors thank Christine Wagner, Eva Scheiffert, Katrin Enderle and Kathrin Stengel for excellent technical assistance.
REFERENCES


TABLES

Table 1:

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
<th>TKI258 (15 mg/kg/d)</th>
<th>TKI258 (30 mg/kg/d)</th>
<th>P-value (control vs. 30 mg/kg/d)</th>
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<tbody>
<tr>
<td>Lymph node metastases</td>
<td>8/10 (80%)</td>
<td>5/10 (50%)</td>
<td>1/9 (11%)</td>
<td>0.0055*</td>
</tr>
<tr>
<td>Liver metastases</td>
<td>6/10 (60%)</td>
<td>3/10 (30%)</td>
<td>1/9 (11%)</td>
<td>0.0573</td>
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</table>

*Fisher's exact test (control vs. 30 mg/kg/d)

Effects of targeting FGFR/PDGFR/VEGFR on liver and lymph node metastases in an orthotopic pancreatic cancer model (treatment started 7 days after tumor cell injection).
FIGURE LEGENDS

Figure 1: Effects of TKI258 on pancreatic cancer cells in vitro. A) Structure of the FGFR/PDGFR/VEGFR inhibitor TKI258 (with friendly permission of Novartis Oncology). B) Western blot of signaling intermediates. Incubation of pancreatic cancer cells with TKI258 for 2 and 20 h led to inhibition of constitutive and FGF-1-induced ERK phosphorylation. C) MTT assay of MiaPaCa2 showed improved growth inhibition upon combination of Gemcitabine and TKI258 after 48 h ($^{\#}P < 0.05$) and 72 h ($^*P < 0.05$). Bars, SEM. D) Migration of pancreatic cancer cells upon stimulation with FGF-1 was significantly induced ($^{\#}P < 0.05$). Treatment with TKI258 impaired constitutive and FGF-1-induced motility after 24 h ($^*P < 0.05$). Bars, SEM. E) Inhibition of FGFR/PDGFR/VEGFR with TKI258 for 24 h diminished expression of N-Cadherin and survivin in a dose-dependent manner. Results are shown for MiaPaCa2, similar results were obtained from BxPC-3 and HPAF-II (except for Fig. 1C).

Figure 2: Impact of TKI258 on endothelial cells in vitro. Western blots of signaling intermediates. A) TKI258 led to inhibition of ERK and c-Raf phosphorylation. Furthermore, activation of FAK and VEGFR upon stimulation with VEGF-A (15 min.) was impaired. B) After stimulation of endothelial cells with FGF-2 (15 min.), TKI258 diminished phosphorylation of ERK and c-Raf. C) Left: In migration assays, VEGF-A significantly induced motility of ECs ($^{\#}P < 0.05$), an effect which was reduced by treatment with TKI258 after 24 h ($^*P < 0.05$). Right: Similarly, TKI258 impaired EC migration upon stimulation with FGF-2 after 24 h ($^*P < 0.05$). Bars, SEM. D) Real-time PCR was used to investigate the effect of FGFR/PDGFR/VEGFR inhibition on DLL4 and survivin expression in ECs; TKI258 (0.5 µM; 24 hours) significantly diminished DLL4 (left) and survivin (right) mRNA ($^*P<0.05$). Bars, SEM.
Figure 3: Effects of TKI258 on PDGF-B induced activation of vascular smooth muscle cells (VSMCs, pericytes). A) Treatment with TKI258 impaired PDGF-B-induced activation of Akt and ERK; STAT3 phosphorylation was also reduced. B) After stimulation of VSMCs with FGF-2 (15 min.), no effects on phosphorylation of ERK and Akt were observed. C) Left: In migration assays, stimulation with PDGF-B significantly increased migration of VSMCs ($P < 0.05$), an effect which was abrogated by treatment with TKI258 (*$P < 0.05$). Bars, SEM.; Right: Similarly, TKI258 impaired VSMC migration upon stimulation with FGF-2 after 24 h. Bars, SEM. D) Real-time PCR was used to investigate the effect of FGFR/PDGFR/VEGFR inhibition on survivin and FGF-2 expression in VSMCs; TKI258 (0.5 µM; 24 hours) significantly diminished survivin (left) and FGF-2 (right) mRNA (*$P < 0.05$). Bars, SEM.

Figure 4: Effects of targeting FGFR/PDGFR/VEGFR in vivo. The effects of TKI258 on tumor growth and angiogenesis were determined in an orthotopic model of pancreatic cancer (L3.6pl). Mice (n = 9–10/group) received either TKI258 (15 or 30 mg/kg/day), or vehicle by oral gavage starting at day seven after tumor cell implantation. A) After 31 days, mice in the TKI258 (30 mg/kg/d) treatment arm showed a significantly reduced tumor burden (tumor weight), compared to mice in the control group (*$P < 0.05$). Treatment with TKI258 (15 mg/kg/d) also showed reduced tumor growth, but this did not reach statistical significance. Bars, SEM. B) Moreover, treatment with 30 mg/kg/d, but not 15 mg/kg/d, TKI258 significantly reduced tumor vascularization in terms of CD31$^+$ vessel area (*$P < 0.05$). Bars, SEM. C) Both dosages of TKI258 significantly impaired tumor cell proliferation in vivo (*$P < 0.05$). Bars, SEM. D) HPAF-II human pancreatic cancer cells were implanted into the subcutis of nude mice. Mice (n = 7/group) received either TKI258 (30 mg/kg/d), or vehicle,
by oral gavage. Blocking FGFR/PDGFR/VEGFR significantly reduced the growth of subcutaneous tumors (*P < 0.01). Bars, SEM.

Figure 5: Impact of TKI258 on growth of established tumors in vivo. A) L3.6pl human pancreatic cancer cells were subcutaneously injected into the right flank of nude mice (n = 5/group). Treatment (TKI258, 30 mg/kg/d) was started when tumors reached a size of 400 mm³. After 4 days of treatment, tumors did not differ in terms of tumor volume, but after 6 days a significant growth inhibition with TKI258 therapy was observed (*P < 0.05). Bars, SEM. B) In the orthotopic model, treatment of established tumor was initiated on day 15 after tumor cell injection (n=8/group). Treatment with TKI258 (30 mg/kg/d) led to a significant increase in survival of mice as shown in the Kaplan-Meier analyses (P < 0.01 vs. control). C) Western blot analyses of tumors from the orthotopic model show a decrease in expression of VEGFR2, PDGFRβ, FGFR3, EGFR, survivin and Hsp90 upon therapy with TKI258. In contrast, expression of PDGFRα was increased compared to controls. D) Treatment with TKI258 led to a significant increase in Tie2 expression in orthotopic tumors (#P < 0.05). Bars, SEM.
Fig. 1:

A

B

TKI258 [1 µM]  FGF-1 (50 ng/ml)

2 h 20 h

-  +  -  +  -  +  -  +

pERK

ERK

β-actin

C

Changes in OD [%]

24 h 48 h 72 h

Gemcitabine [µM]  TKI258 [1 µM]

-  -  1  2  1  2

D

cells/hpf

0 1 2 3 4 5 6 7 8 9

control TKI258 FGF-1 TKI258 + FGF-1

E

TKI258  0.1  0.5  1  [µM]

N-Cadherin

E-Cadherin

Survivin

β-actin
Fig. 2: A VEGF-A (10 ng/ml) TKI258 [0.5 µM] - - + + pERK ERK pc-RafSer289/296/301 pc-RafSer338 c-Raf pFAK FAK pVEGFR2 VEGFR2 β-actin B FGF-2 (50 ng/ml) TKI258 [0.5 µM] - - + + pERK ERK pc-RafSer289/296/301 pc-RafSer338 c-Raf pFAK FAK pVEGFR2 VEGFR2 β-actin C C (left) left (right) cells/hpf VEGF-A TKI258 TKI258 TKI258 TKI258 # * D D (left) left (right) DLL4 mRNA levels normalized to GAPDH control TKI258 TKI258 significance compared to control.*
Fig. 3: A  
PDGF-B (10 ng/ml)  
TKI258 [0.5 µM]  
- - + +  

Akt  
pAkt  
TKI258 [0.5 µM]  
- - + +  
PDGF-B (10 ng/ml)  
++ + +  
TKI258 [0.5 µM]  
- - + +  
FGF-2 (50 ng/ml)  
++ + +  
TKI258 [0.5 µM]  
- - + +  

B  
FGF-2 (50 ng/ml)  
TKI258 [0.5 µM]  
- - + +  

C  
left  
right  

D  
left  
right  

Survivin mRNA levels normalized to GAPDH  
control TKI258  
control TKI258  

FGF-2 mRNA levels normalized to GAPDH  
control TKI258  
control TKI258  
Fig. 4:

A) Tumor weight [g]

B) CD31+ vessel area pixels/hpf

C) BrdU positive cells/hpf

D) Tumor volume [mm³]

control 15 mg/kg/d 30 mg/kg/d

TKI258

two-way ANOVA, Tukey's test: *p < 0.05 compared with control

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**Fig. 5:**

**A**

![Graph showing tumor volume (mm³) over days of treatment](image)

**B**

![Graph showing percent survival over days](image)

**C**

<table>
<thead>
<tr>
<th>control</th>
<th>TKI258</th>
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</thead>
<tbody>
<tr>
<td>VEGFR2</td>
<td></td>
</tr>
<tr>
<td>PDGFRα</td>
<td></td>
</tr>
<tr>
<td>PDGFRβ</td>
<td></td>
</tr>
<tr>
<td>FGFR3</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
</tr>
<tr>
<td>survivin</td>
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</tr>
<tr>
<td>Hsp90</td>
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</tr>
<tr>
<td>β-actin</td>
<td></td>
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</tbody>
</table>

**D**

![Bar graph showing Tie2 mRNA levels](image)
Targeting FGFR/PDGFR/VEGFR impairs tumor growth, angiogenesis and metastasis by effects on tumor cells, endothelial cells and pericytes in pancreatic cancer


Mol Cancer Ther Published OnlineFirst September 1, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-11-0312

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