Dual targeting of Raf and VEGF receptor 2 reduces growth and metastasis of pancreatic cancer through direct effects on tumor cells, endothelial cells, and pericytes

Sven A. Lang,¹ Philipp Schachtschneider,¹ Christian Moser,¹ Akira Mori,¹ Christina Hackl,¹ Andreas Gaumann,² David Batt,³ Hans J. Schlitt,¹ Edward K. Geissler,¹ and Oliver Stoeltzing¹

¹DepartmentsofSurgeryandSurgicalOncologyand²InstituteofPathology,UniversityofRegensburgMedicalCenter,Regensburg,Germany;and³NovartisInstitutesforBiomedicalResearch,Cambridge,Massachusetts

Abstract

The Ras/Raf/MEK pathway represents an important oncogenic signaling pathway in gastrointestinal malignancies, including pancreatic cancer. Although activating B-Raf mutations are infrequent in pancreatic cancer, we hypothesized that targeting Raf could be valuable for therapy of this cancer entity. Moreover, as vascular endothelial growth factor receptor 2 (VEGFR2) is involved in tumor angiogenesis, we sought to investigate the effects of dual inhibition of Raf and VEGFR2 on pancreatic tumor growth, vascularization, and metastasis. Effects of a Raf/VEGFR2 inhibitor (NVP-AAL881) on pancreatic cancer cells, endothelial cells, and vascular smooth muscle cells were determined by Western blotting, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis, and migration assays, respectively. Changes in the expression of VEGF-A or survivin were investigated by ELISA and/or real-time PCR. The growth-inhibitory effects of Raf/VEGFR2 inhibition were additionally evaluated in orthotopic tumor models. Results showed that various Raf isoforms were activated in pancreatic cancer cells and NVP-AAL881 diminished the activation of MEK, Akt, Erk, and also STAT3. Moreover, dual inhibition of Raf/VEGFR2 significantly reduced VEGF expression and impaired cancer cell migration. Importantly, besides blocking VEGF-induced Erk and SAPK phosphorylation in endothelial cells, the Raf inhibitor diminished STAT3 phosphorylation, independent of a VEGF2 blockade, and reduced the expression of survivin. In addition, cell proliferation and migration of both endothelial cells and vascular smooth muscle cells were significantly reduced. In vivo, blocking Raf/VEGFR2 significantly inhibited orthotopic tumor growth and vascularization and reduced cancer metastasis. In conclusion, blocking Raf exerts growth-inhibitory effects on pancreatic tumor cells, endothelial cells, and pericytes and elicits antiangiogenic properties. Dual targeting of Raf and VEGFR2 appears to be a valid strategy for therapy of pancreatic cancer. [Mol Cancer Ther 2008;7(11):3509–18]

Introduction

Treatment of pancreatic cancer remains a great challenge, and development of novel strategies involving molecular targeted therapies has the potential for improving overall survival of patients. Lately, interference with the Ras-Rafmitogen-activated protein kinase (MAPK)-Erk oncogenic signaling pathway has emerged as a promising strategy for cancer therapy, as this pathway is frequently activated by mutation in a variety of human malignancies (1–3). In particular, Raf, which is one of three serine/threonine kinases in the Raf family (A-Raf, B-Raf, and C-Raf) and crucial for MAPK signaling, represents an interesting molecular target. Activating mutations of B-Raf have been reported to occur in the majority of melanomas, where certain single-base missense mutations (V600E) can frequently be detected (2). In pancreatic cancer, Ras-Raf-MAPK signaling is required for mediating growth factor-induced (such as epidermal growth factor) oncogenic effects and activation of this pathway is associated with tumor progression (proliferation) and invasiveness (cell migration; refs. 4, 5). Moreover, it was shown that dual MAPK/epidermal growth factor receptor inhibition significantly decreases growth of KRAS-mutated pancreatic cancer tumors in preclinical models, whereas solely blocking epidermal growth factor receptor (erlotinib) has no growth-inhibitory effect, suggesting that targeting the Ras-Raf-MAPK pathway is superior to anti-epidermal growth factor receptor therapy in KRAS-mutated pancreatic cancer (5). However, activating V600B-Raf mutations are less frequently observed in this cancer entity (6). Nevertheless, disrupting Ras-Raf-MAPK-Erk signaling could prove valuable for therapy of pancreatic cancer, as the oncogene KRAS is frequently mutated in pancreatic cancer, thus leading to an activation of multiple oncogenic signaling cascades (7–9), including the Raf pathway (10). Moreover, certain growth factor activated signaling pathways, such as...
the insulin-like growth factor-I (IGF-I)/IGF-I receptor axis, are in part redundant with or dependent on the Ras-Raf-MEK oncogenic signaling pathway, which leads to complex protumorigenic signaling processes in pancreatic cancer cells (4, 11–13). This may promote not only tumor cell proliferation but also tumor angiogenesis and pancreatic cancer metastasis (11, 14).

One of the predominant systems involved in pancreatic tumor angiogenesis is the endothelial vascular endothelial growth factor receptor 2 (VEGFR2) receptor system, where the ligand VEGF-A is primarily provided by secretion from tumor cells (15, 16). Importantly, a VEGF-induced activation of VEGFR2 promotes migration and proliferation of endothelial cells, two biologic effects required for initiating angiogenesis (17). Moreover, recruitment of pericytes [vascular smooth muscle cells (VSMC)] into tumors, yet another important step in tumor angiogenesis, involves in part the VEGFR and the platelet-derived growth factor receptor (PDGF)-B/PDGF receptor system (18–20). Similarly, this migratory process is in part mediated by activation of MEK/Erk signaling cascades (21, 22). Hence, targeting both endothelial cells and pericytes by blocking VEGFR2, or downstream MEK/Erk signaling, could be a valuable approach for effectively inhibiting tumor angiogenesis and ultimately tumor growth in vivo (23). In addition, results from a recent study also suggest that Raf-1 (C-Raf) represents an interesting target in endothelial cells for reducing cell survival (24). However, the suitability of targeting the Raf-MEK pathway in either endothelial cells or pericytes for reducing tumor angiogenesis or tumor vascularization remains to be further elucidated. This is of particular interest, as dual inhibition of Raf (B-Raf and C-Raf) and VEGFR2 can be achieved by using small-molecule inhibitors, such as NVP-AAL881 (Novartis). NVP-AAL881 is an orally available inhibitor that showed antineoplastic efficacy in preclinical tumor models (25, 26). Importantly, a significant antitumor activity by NVP-AAL881 treatment was observed when tumor cells did harbor mutations in B-Raf (V600E; ref. 27). Nevertheless, the value of using dual Raf/VEGFR2 targeting agents for therapy of pancreatic cancer has not been determined to date.

For the present study, we hypothesized that blocking both Raf and VEGFR2 elicits antiangiogenic and antiangiogenic activity in pancreatic cancer through direct effects on tumor cells, endothelial cells, and pericytes. We used a Raf/VEGFR2 small-molecule tyrosine kinase inhibitor (NVP-AAL881) to determine the effects of such targeted therapy on tumor growth and angiogenesis in experimental models of orthotopic pancreatic cancer in vivo.

Materials and Methods

Cell Culture and Reagents

The human pancreatic cancer cell line HPAF-II was obtained from the American Type Culture Collection, and L3.6pl and BxPC3 cells were kindly provided by Dr. I.J. Fidler (The University of Texas M. D. Anderson Cancer Center). Human umbilical vascular endothelial cells (HUVEC) and VSMC were purchased from Promocell. Cells were cultured in DMEM (Life Technologies) supplemented with 15% FCS and maintained in 5% CO₂ at 37°C as described (11). Recombinant human IGF-I, VEGF-A, PDGF-B, and hepatocyte growth factor (HGF) were purchased from R&D Systems. The Raf/VEGFR2 inhibitor NVP-AAL881 was kindly provided by Novartis and dissolved for in vitro experiments in DMSO (25). For in vivo use, NVP-AAL881 was prepared as described elsewhere (25, 27). The selective VEGFR2 inhibitor Tyrophostin (AG1478) was purchased from Calbiochem (EMD).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays

To evaluate antiproliferative effects of NVP-AAL881 on tumor cells, endothelial cells, and VSMC, cells were seeded into 96-well plates (1 × 10⁵–2 × 10⁵ per well) and exposed to various concentrations of NVP-AAL881. Respective concentrations of DMSO were added to controls accordingly. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to assess cell proliferation and percentages in changes in absorbance were calculated by determining a baseline absorbance reading after an overnight incubation (28).

Western Blot Analyses for Activated Signaling Pathways

Experiments were done in triplicates at a cell density of 60% to 70%. Unless otherwise indicated, cells were incubated with NVP-AAL881 (2 μmol/L) for 24 h before stimulation with either IGF-I (100 ng/mL) or HGF (40 ng/mL). Nonmalignant cells were treated with a lower dose of NVP-AAL881 (1 μmol/L) to reduce cytotoxic effects as determined in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Whole-cell lysates were prepared as described elsewhere (11). Protein samples (50 μg) were subjected to Western blotting on a denaturating 10% SDS-PAGE. Membranes were sequentially probed with antibodies to indicated signaling intermediates. Antibodies to Raf kinases (Raf family sampler kit), phospho-MEK, MEK, phospho-Akt (Thr217Ser473), Akt, phospho-Erk (Thr202/Tyr204), Erk, phospho-STAT3 (Tyr705), and STAT3 were purchased from Cell Signaling Technologies, and antibodies to β-actin were obtained from Santa Cruz Biotechnologies.

Migration Assays

To determine the effect of NVP-AAL881 treatment on cell motility in vitro, migration assays were done using modified Boyden chambers (28, 29). Briefly, 5 × 10⁴ tumor cells were resuspended in 1% FCS-DMEM and seeded into inserts ± IGF-I (100 ng/mL) or HGF (40 ng/mL; Becton Dickinson Bioscience). After 48 h, cells were fixed and migrated cells stained (Diff-Quick reagent; Dade Behring). Cells that migrated through the filters were counted in four random fields and average numbers were calculated. For experiments with HUVEC (±VEGF-A) or VSMC (±PDGF-B), the dose of NVP-AAL881 was reduced to 1 μmol/L and cell migration was determined after 4 to 5 h.

ELISA for VEGF Protein

To determine changes in VEGF secretion by human tumor cells, we used an ELISA kit specific to human
Pancreatic cancer cells were plated at 40% to 50% density and incubated with NVP-AAL881. Analyses of culture supernatants were done according to the manufacturer’s protocol and protein was calculated in picogram per 1,000 viable cells.

**Real-time PCR Analysis for VEGF165 and Survivin Expression**

The effects of Raf inhibition on the expression of VEGF-A or endothelial survivin were determined by real-time PCR. 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 (Qiagen). Selected primer pairs for PCR were as follows: VEGF165 (5’-GCACCCATGGCAGAAGGAGGAG and 3’-AGCCCCCGCATGCATCAG), survivin (5’-GGACCA-CGCCATCTCTCAT and 3’-GACAGAAAGGAGGCACAAC), and β-actin (5’-AGAGGGAAATCGTGCGTGAC and 3’-CAATGTATGACCTGGCGCT). Primers were optimized for MgCl₂ and annealing, and PCR products were confirmed by gel electrophoresis. Real-time PCR was done using the LightCycler system and Roche Fast-Start DNA Master Hybridization Probes master mix (Roche Diagnostics).

**Animal Models**

Eight-week-old male athymic nude mice (BALB/c nu/nu; Charles River) 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 the “Guidelines for the Welfare of Animals in Experimental Neoplasia” published by The United Kingdom Coordinating Committee on Cancer Research. The effects of Raf/VEGFR2 inhibition on the growth of human pancreatic cancer cells (L3.6pl) were investigated in an orthotopic pancreatic cancer model (11). In brief, 1 × 10⁶ human pancreatic cancer cells (L3.6pl) were injected into the pancreatic tail of mice. After implantation, tumors were allowed to grow 7 days before treatment was initiated. Mice were randomized into groups (n = 5-6 per group) receiving either vehicle (controls) or NVP-AAL881 (100 mg/kg/d) by oral gavage. The dose of NVP-AAL881 was chosen based on previous reports and in the intention not to use this compound at the maximal tolerated dose (25, 27). In this first model, mice were sacrificed on day 21 after tumor cell inoculation and excised tumors were measured and weighed. The compound was well tolerated and no significant loss in body weight occurred in studies. For immunohistochemical analyses, tumors were embedded in OCT solution. The effects of Raf/VEGFR2 inhibition on pancreatic cancer metastasis were investigated in a second orthotopic tumor model (L3.6pl) using fewer cells per implantation (250,000 per 50 μL) to extend the overall duration of the experiment, thus allowing metastases formation. In analogy to the first study, mice (n = 9-10 per group) were randomized to treatment groups and the experiment was terminated on day 33 after cell implantation. The incidence of liver and lymph node metastases was determined and pancreatic tumors excised and weighed.

**Immunohistochemical Analysis of Tumor Vasculatization**

Multiple cryosections were obtained from tumors for all immunohistochemical analyses. CD31⁺ vessel area was assessed using rat anti-mouse CD31/PECAM-1 antibody (Pharmingen) and peroxidase-conjugated goat anti-rat IgG (Jackson Research Laboratories) as described previously (11). 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 ×40 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 (11, 28).

**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. Tumor-associated variables of in vivo experiments were tested for statistical significance using the Mann-Whitney U test for nonparametric data. The two-sided Student’s t test was applied for analysis of in vitro data. Fisher’s exact test was applied to test for significance of incidence of metastases formation. All results are expressed as the mean ± SE.

**Results**

**Effects of Raf Inhibition on Signaling Pathway Activation in Human Pancreatic Cancer Cells**

Because activating Rac mutations occur rarely in pancreatic cancer (6, 30, 31), we first determined by Western blotting whether B-Raf and other Raf isoforms are activated in human pancreatic cancer cell lines. Moreover, as the tumor microenvironment also consists of tumor endothelial cells and pericytes that eventually could be affected by anti-Raf targeted therapy, the expression and activation of Raf kinase isoforms was additionally determined in these cell types. Results show that various Raf kinase isoforms are predominantly expressed and activated in pancreatic cancer cells; however, overall activation of C-Raf might vary among cell lines, as an “inhibitory” phosphorylation of C-Raf (Ser²⁵⁹) was also detectable in cancer cells (Fig. 1; ref. 32). Interestingly, endothelial cells also showed a detectable phosphorylation of Raf isoforms (Fig. 1). In contrast, VSMC that were used as representative cell type for pericytes did not express comparably high levels of either Raf or phospho-Raf.

In view of the fact that tumor cells appear to be the primary target of an anti-Raf therapy, the effects of Raf inhibition on the activation of signaling cascades in
Pancreatic cancer cells were investigated using the Raf inhibitor NVP-AAL881, which is known to inhibit B-Raf and C-Raf (25). Moreover, as certain growth factors, such as IGF-I and HGF (scatter factor), are involved in the growth and angiogenesis of pancreatic cancer, recombinant proteins were used for stimulating cancer cells in vitro (11, 33, 34). Treatment of pancreatic cancer cells (HPAF-II) with NVP-AAL881 substantially impaired IGF-I-mediated signaling in terms of reducing the activation of MEK, Erk, and STAT3 (Fig. 2A). In addition, NVP-AAL881 diminished a HGF-induced phosphorylation of MEK and Akt (Fig. 2B). However, substantial changes in Erk activation were not detectable, suggesting that the HGF/c-Met signaling cascade is only partially redundant with the Ras-Raf-MEK pathway. The effects of NVP-AAL881-mediated impaired IGF-I and HGF signaling were additionally confirmed in other pancreatic cancer cell lines (L3.6pl and BxPC3), showing robust inhibition of MEK, Erk, Akt, and STAT3 signaling intermediates (data not shown). Overall, inhibition of these pathways by NVP-AAL881 led to a significant reduction in VEGF secretion by tumor cells (Fig. 2C). The changes in VEGF expression were additionally confirmed on mRNA level (data not shown). We conclude from these experiments that inhibition of Raf effectively interferes with multiple growth factor-induced signaling pathways and diminishes VEGF expression in human pancreatic cancer cells.

**Effects of Raf Inhibition on Cancer Cell Migration**

The consequences of a potent inhibition of oncogenic signaling pathways by NVP-AAL881 were further evaluated in migration assays, where either recombinant human IGF-I or HGF served as a chemoattractant. Importantly, these pancreatic cancer cell lack VEGFR2 expression. Results show that both growth factors significantly induced cancer cell migration in vitro (Fig. 3A and B). Treatment with the Raf inhibitor NVP-AAL881 significantly reduced pancreatic cancer cell migration induced by either IGF-I (Fig. 3A) or HGF (Fig. 3B). However, the promigratory effects of HGF were impaired to a lesser degree compared with the inhibition seen when IGF-I served as a chemoattractant (Fig. 3B). Results from these experiments suggest that the strategy of blocking Raf activity in pancreatic cancer cells harbors the potential to reduce cancer metastasis in vivo.

**Figure 1.** Expression and activation of Raf isoforms in endothelial cells, pericytes, and pancreatic cancer cells. Western blot analysis was done to determine phosphorylation and expression of Raf kinase isoforms in HUVEC, VSMC, and human pancreatic cancer cell lines (BxPC3, HPAF-II, and L3.6pl). Activation of Raf kinases appeared to be higher in pancreatic cancer cells compared with endothelial cells or pericytes. Probing for β-actin served as a loading control.

**Figure 2.** Effect of Raf inhibition on signaling pathway activation in human pancreatic cancer cells. The effects of NVP-AAL881 on constitutive and inducible phosphorylation of signaling intermediates were determined by Western blotting. Because growth factors IGF-I and HGF are implicated in pancreatic cancer growth and metastasis, recombinant proteins were used for stimulating cells after a preincubation with NVP-AAL881 (2 μmol/L, 24 h). A, treatment with NVP-AAL881 impaired IGF-I-mediated (100 ng/mL) signaling in pancreatic cancer cells (HPAF-II) in terms of reducing the activation of MEK, Erk, and STAT3 (Fig. 2A). In addition, NVP-AAL881 diminished a HGF-induced phosphorylation of MEK and Akt (Fig. 2B). However, substantial changes in Erk activation were not detectable, suggesting that the HGF/c-Met signaling cascade is only partially redundant with the Ras-Raf-MEK pathway. The effects of NVP-AAL881-mediated impaired IGF-I and HGF signaling were additionally confirmed in other pancreatic cancer cell lines (L3.6pl and BxPC3), showing robust inhibition of MEK, Erk, Akt, and STAT3 signaling intermediates (data not shown). Overall, inhibition of these pathways by NVP-AAL881 led to a significant reduction in VEGF secretion by tumor cells (Fig. 2C). The changes in VEGF expression were additionally confirmed on mRNA level (data not shown). We conclude from these experiments that inhibition of Raf effectively interferes with multiple growth factor-induced signaling pathways and diminishes VEGF expression in human pancreatic cancer cells.

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independent experiments. Aminedat2 Aadose of1 substantially reduced constitutive STAT3 phosphorylation intermediates (Fig. 4A). Importantly, blocking Raf/VEGFR2 markedly reduced phosphorylation of these activation of Erk and SAPK signaling intermediates and stimulation of endothelial cells with VEGF-A led to an reduce direct cytotoxic effects. In Western blot analysis, effects of Raf inhibition on cancer cell migration were determined in in vitro assays where either IGF-I (100 ng/mL) or HGF (40 ng/mL) served as a chemo-attractant. A, IGF-I significantly induced migration of HPAF-II cells (**, P < 0.01). Treatment with NVP-AAL881 significantly impaired basal (**, P < 0.01) and IGF-I-induced cancer cell migration compared with respective controls (**, P < 0.01). B, similarly, HGF led to significantly enhanced promigratory properties (#, P < 0.01). Blocking Raf markedly lowered promigratory effects of HGF (*, **, P < 0.01). Mean ± SE of three independent experiments.

**Effects of Raf/VEGFR2 Inhibition on Endothelial Cells and Pericytes**

Because NVP-AAL881 is a dual inhibitor of Raf and VEGFR2, its effects were next determined on HUVEC, which yet represent another target of NVP-AAL881 therapy in pancreatic tumors. By 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis, we first determined antiproliferative effects of NVP-AAL881 on endothelial cells in vitro. NVP-AAL881 elicited significant growth-inhibitory effects on endothelial cells, with an IC50 determined at 2 μmol/L (72 h; Supplementary Fig. S1). However, a dose of 1 μmol/L was selected for in vitro experiments to reduce direct cytotoxic effects. In Western blot analysis, stimulation of endothelial cells with VEGF-A led to an activation of Erk and SAPK signaling intermediates and NVP-AAL881 markedly reduced phosphorylation of these intermediates (Fig. 4A). Importantly, blocking Raf/VEGFR2 substantially reduced constitutive STAT3 phosphorylation in endothelial cells (Fig. 4A). To define whether this reduction in constitutive STAT3 activation is mediated in part via blocking VEGFR2 or through other kinases, a selective VEGFR2 inhibitor was used (Tyrphostin AG1478). Incubation of endothelial cells (in the absence of VEGF in media) with a VEGFR2 inhibitor dose-dependently reduced basal phosphorylation of VEGFR2 and Erk (Fig. 4B). In contrast, the constitutive activation of STAT3 remained unaffected, suggesting that the NVP-AAL881-mediated reduction of constitutive STAT3 in endothelial cells is not sustained via VEGFR2. However, stimulation of endothelial cells with VEGF led to an increase in STAT3 phosphorylation, an effect that was dose-dependently diminished by VEGFR2 inhibition with Tyrphostin (Supplementary Fig. S2), hence implying that inducible STAT3 activation is mediated via VEGF/VEGFR2, whereas basal STAT3 activation is susceptible to Raf inhibition.

Because the STAT3 pathway has been identified to be important for regulating endothelial cell survival in terms of modulating the expression of survivin (35, 36), we sought to investigate whether NVP-AAL881 could alter survivin expression in endothelial cells. As determined by real-time PCR, Raf inhibition indeed down-regulated survivin mRNA in endothelial cells (Fig. 4C). Moreover, inhibition of cell signaling by NVP-AAL881 (1 μmol/L, 4 h) also significantly abrogated promigratory effects of VEGF-A in migration assays (Fig. 4D). Results from these experiments suggest that targeting Raf, in addition to VEGFR2, exerts antiangiogenic activity through modulating survivin expression and cell motility.

Although pericytes (VSMCs) did not express high levels of activated Raf, we next determined the effects of NVP-AAL881 on cell signaling in VSMCs. Due to the fact that PDGF-B is the predominant growth factor involved in pericyte recruitment, this growth factor was used for stimulating cells in vitro. By Western blot analysis, dual inhibition of Raf and VEGFR2 substantially disrupted signaling in pericytes in terms of reducing the PDGF-B-induced phosphorylation of MEK, Erk, and STAT3 (Fig. 5A). Moreover, NVP-AAL881 not only reduced proliferation of VSMCs in a dose-dependent manner (Fig. 5B) but also significantly diminished pericyte migration in response to PDGF-B (5 h; Fig. 5C). We conclude from these experiments that inhibition of Raf elicits additional antiangiogenic activity through direct effects on pericytes.

**Effect of Dual Raf and VEGFR2 Inhibition on Orthotopic Pancreatic Tumor Growth and Vascularization**

To estimate the effects of targeting both Raf kinase and VEGFR2 on tumor growth and angiogenesis in vivo, we used an orthotropic pancreatic tumor model (L3.6pl). After 21 days, therapy with NVP-AAL881 (100 mg/kg/d) led to a significant reduction in orthotopic tumor growth as reflected by the final tumor weights of excised tumors (Fig. 6A). Similar significant growth inhibitory effects of NVP-AAL881 were detected in a s.c. xenografted tumor model using a second cancer cell line (HPAF-II; data not shown).

Furthermore, blocking Raf and VEGFR2 led to a significant reduction in tumor vascularization as expressed
by decreased CD31+ vessel areas in pancreatic tumors of the treatment group (Fig. 6B). In addition, PCR analysis of tumor tissues revealed a substantial decrease in VEGF mRNA in NVP-AAL881-treated tumors (Supplementary Fig. S3). 5 In a second (prolonged) orthotopic tumor model (n = 9-10 per group), we detected not only a significant growth inhibition by blocking Raf/VEGFR2 (mean ± SE tumor weights: control 2.3 ± 0.3 g versus NVP-AAL881 1.3 ± 0.3 g; P < 0.05) after 33 days but also a significant reduction in the occurrence of lymph node metastases and marked inhibition of liver metastases formation (Table 1).

Together, these results indicate that simultaneous targeting of Raf and VEGFR2 could be used to effectively inhibit growth, metastasis, and vascularization of human pancreatic cancer, a biological effect that appears to be mediated via a combination of direct antiangiogenic and antineoplastic properties of an anti-Raf/VEGFR2 therapy.

Discussion
Our study shows that dual targeting of Raf and VEGFR2 elicits potent growth inhibitory effects in an experimental model of pancreatic cancer, which is mediated through direct effects on cancer cells, endothelial cells, and pericytes. Importantly, we identified Raf to be a valid target in endothelial cells, as inhibition of this kinase substantially reduces endothelial MEK/Erk and STAT3 activation, survivin expression, cell migration, and cell proliferation. Moreover, the Raf inhibition also affected VSMC (pericytes) in terms of disrupting PDGF-B signaling (MEK/Erk), reducing STAT3 activation, and abrogating promigratory effects, which suggests that blocking Raf per se elicits direct antiangiogenic effects. Hence, interference with the Ras-Raf-MEK plus the VEGFR2 pathway harbors the potential to improve efficacy of antiangiogenic/antineoplastic therapy regimens in the treatment of pancreatic cancer.

The strategy of inhibiting the oncogenic Raf signaling pathway for cancer therapy has recently gained attention (3, 37). However, targeting the B-Raf kinase isoform appears to be most effective when activating mutations are present in cancer cells, such as the reported V600E-Raf mutation in thyroid cancer and melanoma (27, 37). In our study, we investigated the effects of a small-molecule inhibitor to Raf and VEGFR2 on pancreatic cancer cells, although the Raf signaling cascade in this cancer entity is predominantly activated via KRAS (10), an oncogene known to be mutated in >90% of pancreatic adenocarcinomas (38, 39). Indeed, we found that inhibition of Raf led to a potent reduction in MEK, Erk, and STAT3 activation in...
cancer cells, suggesting that the Raf pathway represents a valid molecular target in this cancer entity. We also observed that Akt phosphorylation was diminished on exposure to NVP-AAL881, which we speculate to be an effect mediated through a broader range of kinase inhibition by NVP-AAL881 beyond Raf and VEGFR2 (25, 40). Importantly, in an initial analysis for constitutive phosphorylation of Raf family kinases, we found that all three kinases (A-Raf, B-Raf, and C-Raf) are phosphorylated in pancreatic cancer cells, which could imply that pan inhibitors to Raf might even be more effective than solely blocking one or two Raf isoforms. However, the role of C-Raf remains uncertain, as we also detected a constitutive inhibitory phosphorylation at Ser\textsuperscript{259} as suggested in a study by Zimmermann and Moelling (32). Currently, most small-molecule Raf inhibitors display activity predominantly against C-Raf and to a lesser extent to B-Raf (25, 26, 41), whereas the compound NVP-AAL881, which we used for experiments, has reported activity against both B-Raf and C-Raf kinases (25). Nevertheless, a new generation of compounds will be required to determine a potential superiority of pan-Raf inhibitors in pancreatic cancer. Importantly, the Raf-inhibitory effect against particular kinase isoforms varies greatly between Raf targeting substances, thus demanding that the therapeutic efficacy is always to be determined in the appropriate preclinical

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**Figure 5.** Effects of Raf inhibition on PDGF-B-induced signaling pathway activation in VSMC. Western blotting was done to determine the effects of NVP-AAL881 (1 \( \mu \)mol/L) on PDGF-B-induced (10 ng/mL) cell signaling in VSMCs (pericytes). **A**, inhibition of Raf substantially diminished PDGF-B-induced phosphorylation of MEK, Erk1/2, and STAT3. **B**, antiproliferative effects were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis, showing that NVP-AAL881 significantly reduced cell proliferation after 72 h (#, \( P < 0.01 \) versus control). Moreover, this antiproliferative effect was even more pronounced when PDGF-B was present in the medium (*) \( P < 0.01 \) versus PDGF-B). **C**, in migration assays, stimulation with PDGF-B significantly increased migration of VSMCs (#, \( P < 0.01 \) versus PDGF-B), an effect that was abrogated by treatment with NVP-AAL881 (**, \( P < 0.01 \)). Inhibition of Raf also reduced basal migratory properties of VSMCs (*, \( P < 0.01 \)). Mean \( \pm \) SE of three independent experiments.
In our study, the compound NVP-AAL881 significantly reduced tumor cell proliferation in vitro and growth of pancreatic tumors in vivo; hence, our results are in favor of a strategy of targeting B-Raf/C-Raf for therapy of pancreatic cancer.

In addition, the VEGFR2 function was also blocked by the NVP-AAL881 compound, which we speculate to be an important component of the antineoplastic efficacy of this therapy concept, as VEGFR2 represents one crucial promoter of tumor angiogenesis in pancreatic cancer (15). Nevertheless, clinical trials solely targeting the VEGF/VEGFR2 pathway (bevacizumab) did thus far not provide convincing evidence for efficacy of such angiogenic approach. Importantly, Ras signaling in endothelial cells can be triggered by multiple proangiogenic receptors, including VEGFR2, fibroblast growth factor receptor-2, Tie2 (Tek), integrins (αvβ3 and αvβ5), and EDG1 (23, 42, 43). Therefore, it is not yet clear whether only VEGFR2, Raf, or both kinases are effectively targeted by NVP-AAL881 to achieve the inhibition of angiogenesis. Importantly, we also detected direct effects of NVP-AAL881 on endothelial cells (HUVEC) in terms of MEK and STAT3 signaling inhibition that appeared to be independent of VEGFR2. This is the first study to show that treatment with NVP-AAL881 (inhibition of Raf-MEK) leads to a robust inhibition of constitutive and inducible STAT3 activation in endothelial cells. This is of particular importance because both the Raf-MEK pathway and STAT3 have been implicated in the regulation of endothelial cell survival by modulating the expression of survivin. The involvement of Raf-MEK in survivin expression has very recently been shown in an experimental study by Kumar et al., showing that endothelial cell Bcl-2 expression significantly increased the expression of survivin via activation of MEK and thereby diminishing the proapoptotic effects of γ-radiation (24). Importantly, endothelial STAT3 phosphorylation appears to be one crucial downstream signaling component for up-regulating survivin expression in endothelial cells, which has been shown by studies that used either a dominant-negative STAT3 construct or cytokines for activating STAT3 (35, 36). Moreover, the Raf-1 kinase mediates basic fibroblast growth factor-induced antiapoptotic and proangiogenic effects on endothelial cells, suggesting that Raf kinases per se could serve as a valid target for antineoplastic therapy (44). This has been shown in a study by Alavi et al., which provided evidence that apoptosis signal-regulating kinase 1 of the MAPK kinase family forms a complex with Raf-1, which in turn is required for endothelial cell apoptosis in response to the genotoxic chemotherapeutic agent doxorubicin. In addition, the authors showed that basic fibroblast growth factor, but not VEGF, neutralizes the death-promoting activity of apoptosis signal-regulating kinase 1 through an interaction with Raf-1 (44). We therefore propose that targeting both VEGFR2 and Raf is an effective strategy for reducing angiogenic effects of basic fibroblast growth factor and VEGF on tumor-associated endothelial cells in pancreatic cancer.

The antiangiogenic properties of dual targeting Raf/VEGFR2 is further supported by our findings in VSMC, which were used for investigating effects on NVP-AAL881 on pericytes. Because pericyte recruitment represents another important step in tumor angiogenesis that involves MEK signaling (21, 45, 46), we hypothesized that...
Raf/VEGFR2 inhibition could also affect cell signaling and cell function. Indeed, we found that NVP-AAL881 substantially inhibited the proliferation and PDGF-B-induced cell migration of VSMCs. The inhibitor diminished PDGF-B-mediated MEK activation and STAT3 phosphorylation, although we did not detect strong levels of B-Raf expression by Western blotting. Nevertheless, both Ras and c-Raf-1 have been shown to be functionally relevant in VSMCs for regulating cell proliferation and survival (47–49), suggesting that NVP-AAL881 could also exert inhibitory effects on Raf-1 (C-Raf), as VSMCs and pericytes have reportedly no detectable VEGFR2 expression (50). Again, this observation suggests that dual targeting of Raf kinases and VEGFR2 could prove valuable for improving antiangiogenic efficacy of antineoplastic therapy concepts in cancer treatment.

In conclusion, targeting Raf and VEGFR2 may lead to an effective inhibition of growth and metastasis of pancreatic cancer. Together with its additional antiangiogenic properties in terms of disrupting MEK and STAT3 activation in endothelial cells and VSMCs, which translated into potent inhibition of cell migration, proliferation, and survivin expression, inhibitors to Raf/VEGFR2 might be a valuable addition to antineoplastic chemotherapy regimens or multimodality therapies for treating pancreatic cancer. Hence, these results provide are in favor of a rationale using dual inhibitors to Raf and VEGFR2 in clinical trials in pancreatic cancer.

Disclosure of Potential Conflicts of Interest

D. Batt, employee of Novartis Institute. No other potential conflicts of interest were disclosed.

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References

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Dual targeting of Raf and VEGF receptor 2 reduces growth and metastasis of pancreatic cancer through direct effects on tumor cells, endothelial cells, and pericytes

Sven A. Lang, Philipp Schachtschneider, Christian Moser, et al.

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