Nintedanib Is a Highly Effective Therapeutic for Neuroendocrine Carcinoma of the Pancreas (PNET) in the Rip1Tag2 Transgenic Mouse Model

Ruben Bill, Ernesta Fagiani, Adrian Zumsteg, Helena Antoniadis, David Johansson, Simon Haefliger, Imke Albrecht, Frank Hilberg, and Gerhard Christofori

Abstract

Purpose: Pancreatic neuroendocrine tumors (PNET) represent a rare but challenging heterogeneous group of cancers with an increasing incidence over the last number of decades. Herein, we report an in-depth evaluation of the new antiangiogenic small-molecule tyrosine kinase inhibitor (TKI) nintedanib in the preclinical Rip1Tag2 transgenic mouse model of neuroendocrine carcinoma of the pancreas (insulinoma).

Experimental Design: We have assessed the antiangiogenic and antitumor activity of nintedanib, in comparison with other antiangiogenic TKI, by treating Rip1Tag2 transgenic mice with different treatment schedules complemented with histopathologic, cell biologic, and biochemical analyses.

Results: Prolonged nintedanib treatment of Rip1Tag2 mice has led to a strong suppression of angiogenesis, accompanied by a reduced tumor burden, which translated into a significant prolongation of survival. Despite nintedanib's inhibitory action on perivascular cells, the blood vessels remaining after therapy displayed a considerably mature phenotype with tight perivascular cell coverage and preserved perfusion. Nintedanib treatment did not increase local tumor invasiveness or metastasis to the liver and pancreatic lymph nodes—a phenomenon that has been observed with antiangiogenic treatments of Rip1Tag2 transgenic mice in other laboratories. In contrast with the strong reduction in blood microvessel densities, nintedanib did not have any impact on tumor lymphangiogenesis.

Conclusions: Based on our findings, we propose the clinical evaluation of the antiangiogenic drug nintedanib as a new treatment modality for PNET patients, notably in a direct comparison with already established therapeutic regimens, such as sunitinib.

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Introduction

Pancreatic neuroendocrine tumors (PNET), although representing a minority of pancreatic tumors, remain a therapy-challenging heterogeneous group of tumors with increasing incidence over the last decades (1). Whereas 45% to 60% of PNETs are nonfunctional, 40% to 55% produce a variety of hormones leading to different clinical presentations, e.g., hypoglycemic syndrome in the case of insulin-producing PNETs (insulinoma; ref. 2). Aside from cytoreductive surgery, somatostatin analogues, peptide receptor-targeted radiotherapy, and systemic chemotherapy, management of advanced PNETs involves targeted therapies, such as the mTOR inhibitor everolimus or the antiangiogenic small-molecule tyrosine kinase inhibitor (TKI) sunitinib (2). In a phase III clinical trial, sunitinib was shown to be highly effective and significantly prolonged progression-free and overall survival of PNET patients. This study even had to be discontinued early, because of a significantly worse clinical outcome in the placebo group. However, a number of patients experienced treatment-related side effects, such as grade 3 or 4 hypertension and neutropenia in 10% and 12% of patients, respectively (3). Reducing adverse events is of particular importance in this cancer entity, since patients usually undergo long-term therapy and usually experience good quality of life even without treatment until late in the course of the disease (4).

Despite the encouraging results derived from numerous preclinical studies, antiangiogenic therapies targeting mainly the VEGF/VEGF receptor axis have widely failed to substantially increase patient survival in a large number of cancer types (5). In mouse models, the upregulation of alternative proangiogenic factors, such as FGFs, PDGFs, Bv8, and others, has been shown to mediate the resistance to blocking the VEGF-A/VEGFR2 axis. Hence, a simultaneous targeting of the VEGF and the FGF receptor families and other alternative signaling pathways may lead to an improved clinical outcome (6–9). Nintedanib (BIBF1120), a small-molecule kinase inhibitor that targets not only VEGF and PDGF receptors but also FGF receptors and c-Src (10, 11), has recently been shown to yield significant anticancer effects in a variety of preclinical cancer models and in non–small cell lung cancer (NSCLC) patients (10, 12, 13). In a recent phase III clinical trial of nintedanib in NSCLC patients (LUME-Lung 1), the
Translational Relevance
Pancreatic neuroendocrine tumors are often diagnosed at an advanced stage and thus remain a deadly disease with restricted systemic treatment options. Nevertheless, this group of cancers in advanced stages has proven to be sensitive to the antiangiogenic tyrosine kinase inhibitor sunitinib—yet with considerable side effects. On the other hand, the broad-spectrum antiangiogenic tyrosine kinase inhibitor nintedanib has displayed an encouraging anticaner and safety profile in preclinical cancer models and in cancer patients. We have employed the Rip1Tag2 transgenic mouse model of hormone-positive neuroendocrine carcinoma of the pancreas to test the efficacy of nintedanib in treating insulinoma in a preclinical setting. This mouse model recapitulates multistage neuroendocrine carcinogenesis and the co-evolution of the tumor microenvironment with the tumor cells. Notably, it has been shown to be highly predictive in translating preclinical drug evaluation into successful clinical application, e.g., as demonstrated in the cases of sunitinib and mTOR inhibition.

The majority of side effects ascribed to nintedanib were of gastrointestinal origin (diarrhea, nausea, vomiting) and reversible elevations in liver enzymes. Hypertension and white blood cell alterations were not pronounced in the cohort treated with nintedanib plus standard chemotherapy versus the cohort treated with chemotherapy alone (13). Moreover, treatment of idiopathic pulmonary fibrosis in a randomized, double-blind phase III trial has not resulted in any severe adverse effects (14).

The apparent discrepancies between the efficacies of antiangiogenic therapies in preclinical models and in patients may be attributed to the altered stromal microenvironment that human tumor cells encounter in immunodeficient mice, frequently aggrivated by an inadequate subcutaneous (heterotopic) implantation of cancer cells of other organs. Especially when evaluating drugs that mainly target the tumor microenvironment, as is the case with antiangiogenic therapies, these artifacts might be of significant importance (10, 15). A solution to this problem is the use of transgenic animal models that stochastically develop endogenous tumors in a robust and reproducible manner. For example, the Rip1Tag2 transgenic mouse model of PNET expresses the oncogenic SV40 Large T antigen (Tag) under the control of the rat insulin promoter (Rip) and reproducibly elicits multistage tumorigenesis of the insulin-producing β cells of the pancreas (16, 17). The stochastic and stepwise development enables the tumor microenvironment to co-evolve with the cancer cells, thus recapitulating the patient situation. Indeed, the Rip1Tag2 transgenic model has proven a versatile and robust preclinical model that reliably mimics human insulinoma. For example, SV40 Tag binds and inactivates the retinoblastoma (RB) and the p53 tumor-suppressor gene products, which are found dysregulated in a large subset of human PNETs as well (18, 19). Rip1Tag2 mice develop PNETs in a multistep fashion. Although SV40 Large T antigen serves as the initial oncogenic driver presumably in all β cells present within the islets of Langerhans of the pancreas, stochastically occurring additional genetic alterations lead to hyperplastic lesions in only some of the islets. A proportion of these preneoplastic lesions further progress and induce the formation of new blood vessels (angiogenic switch). Only few of these angiogenic hyperplastic islands progress first to adenoma and subsequently become invasive (carcinoma; refs. 16, 17). The Rip1Tag2 transgenic mouse model has been used for the testing of numerous experimental therapies and has been instrumental in predicting the successful application of novel therapies in clinical trials (20). For example, preclinical results derived from combined anti-VEGFR and anti-PDGFRB treatments of the Rip1Tag2 transgenic PNET mouse model have paved the way for the successful clinical application of sunitinib in PNET patients (3, 21, 22). Finally, the use of Rip1Tag2 transgenic mice has led to the use of radiolabeled Exendin-IV to noninvasively image insulinoma in patients, a method that is now in clinical use (23).

Here, we report an in-depth preclinical characterization of nintedanib in the Rip1Tag2 transgenic mouse model of neuroendocrine carcinoma of the pancreas. We report a strong antiangiogenic response that translates into significantly reduced tumor growth and prolonged survival. Despite a vast reduction in microvesSEL density upon extended nintedanib treatment, only a slight increase in tumor hypoxia was observed. In addition, the remaining tumor blood vessels displayed mature characteristics. Furthermore, we did not find increased local invasiveness or metastases in the liver and lymph nodes in any of the treatment regimens assessed. Based on the results, we propose that nintedanib should be evaluated in clinical trials as a new treatment modality of PNET.

Materials and Methods

Mice
The generation and characterization of Rip1Tag2 transgenic mice have been reported elsewhere (16). Mice were kept in a C57Bl/6 genetic background. Starting from week 9 of age, mice were fed with food pellets supplemented with 60% glucose (Provimi Kliba AG) to counteract detrimental hypoglycemia caused by excessive insulin production. All animal experiments were performed according to the guidelines and legislation of the Swiss Federal Veterinary Office (SFVO) and the Cantonal Veterinary Office, Basel-Stadt, Switzerland, under license numbers 1878 and 1908.

Therapy studies
Both female and male Rip1Tag2 transgenic mice were treated as indicated in the respective figure legends, starting from 9 to 10 weeks of age (early stage disease) or from 11 weeks of age (late stage disease). PTK,ZK222584 (PTK/ZK; provided by Novartis Pharma) was dissolved in polyethylene glycol 300 (PEG300; Sigma) and administered daily by oral gavage at 100 mg/kg body weight. Nintedanib (provided by Boehringer Ingelheim) was dissolved in Hydroxethylcellulose Natrosol 0.5% (vehicle treatment; Boehringer Ingelheim) and administered daily by oral gavage at 50 mg/kg body weight. Sunitinib L-malate (LC Laboratories) was formulated in carboxymethylcellulose vehicle as described elsewhere (control treatment; ref 22) and administered daily by oral gavage at 40 mg/kg body weight.

Total tumor volume per mouse was extrapolated by measuring the diameter (d) of single macroscopic tumors, employing the formula volume = \(\frac{4}{3}\pi \left(\frac{d}{2}\right)^3\) and summing up the volumes of individual tumors per mouse.

In the survival study, mice were euthanized by CO₂ suffocation prior to death due to hypoglycemia, according to defined...
termination criteria. The termination criteria were based on an activity score (normal activity = score 0; wiggling/reduced activity = 2; still/hunchback/poor general condition = 3) and blood glucose levels (>3.2 mmol/L = score 0; 1.1–2.0 mmol/L = 1; 0.7–1.0 mmol/L = 2; <0.7 mmol/L = 3) measured using the blood glucose meter Contour Next (Bayer). Mice were euthanized when reaching a total score of 4 or if presenting with score 3 in one of the two criteria.

**Hypoxia and vessel functionality**

To detect hypoxic tumor areas, pimonidazole HCl (Hypoxyprobe Omni Kits; Hypoxyprobe, Inc.) at 60 mg/kg was injected i.p. 2 hours prior to euthanizing the animals. Reduced pimonidazole in hypoxic tumor regions was visualized by immunofluorescence staining with a rabbit anti-pimonidazole antisera (Hypoxyprobe Omni Kits; Hypoxyprobe, Inc.)

Leaky blood vessels were detected by injecting 250 µg fluorescein-labeled dextran (70 kDa; Life Technologies; D-1822) in 200 µL PBS intravenously via the tail vein. After a circulation time of 5 minutes, terminally anesthetized mice were first perfused via the left cardiac ventricle with PBS and subsequently with PBS/4% paraformaldehyde (PFA).

For the detection of patent blood vessels, 100 µg of fluorescein-labeled lycopersicon esculentum (tomato) lectin (Vector Laboratories; GL-1171) was injected in 100 µL PBS intravenously via the tail vein. After a circulation time of 4 minutes, terminally anesthetized animals were perfused with PBS/4% PFA followed by PBS via the left cardiac ventricle.

**Tissue preparation for histology**

For hematoxylin & eosin (H&E), immunohistochemistry (IHC), and immunofluorescence (IF) staining, organs (pancreas and liver) were isolated, fixed overnight in PBS/4% PFA at 4°C, dehydrated with ethanol/xylene, and subsequently embedded in paraffin. For IF stainings, pancreata were fixed during 2 hours in PBS/4% PFA and cryopreserved in PBS/20% sucrose overnight, both at 4°C. Pancreata were embedded, snap frozen in optimal cutting temperature (OCT) freezing solution (Thermo Scientific), and stored at −80°C. Macroscopic images of whole dissected pancreata were acquired using a Nikon D5000 camera with AF-S Micro Nikkor 105 mm f/2.8D lens.

**Immunohistochemistry and immunofluorescence**

See Supplementary Information.

**Histopathologic grading**

H&E staining of 5-µm-thick formalin-fixed paraffin-embedded pancreas sections was performed as described (24). Histopathologic analysis, i.e., grading, was conducted on H&E-stained paraffin sections in a blinded manner. Grading was performed as previously described (25). In brief, tumors were categorized into either noninvasive insulinoma with smooth tumor borders (adenoma), into tumors with no more than 1 to 2 microinvasions (IC1), or into macroinvasive carcinomas (IC2), including rare anaplastic carcinomas (Supplementary Fig. S1A). All tumors per experimental group were pooled and percentages of the respective grade are indicated.

**Tumor cell viability assay**

Two × 10⁶ primary insulinoma tumor cells/well were seeded in a 96-well plate 3 days (day −3) prior to the addition of nintedanib dissolved in DMSO at different concentrations (day 0). At day +2, nintedanib and medium were replaced. At day +4, cells were fixed with 4% PFA/PBS, blocked, and permeabilized with 0.1% BSA and 0.1% Triton X-100/PBS. To identify intact cells, nuclei and the microtubule cytoskeleton were stained with Hoechst and a rat anti-tubulin (YLI/2) antibody (Santa Cruz; sc-33029; 1:1,000), respectively, followed by the incubation with an anti-rabbit Alexa488-labeled secondary antibody (Molecular Probes; 1:400). Images were acquired employing an Operetta High Content Imaging System (PerkinElmer; Objective 20xWD; non-confocal acquisition).

**Statistical methods**

Data were analyzed and graphs were generated with GraphPad Prism 6 (GraphPad Prism Software Inc.) or R version 2.15.1 (The R Project for Statistical Computing).

**Results**

Nintedanib efficiently reduces tumor burden and prolongs survival

We first investigated the gross effects of nintedanib on tumor growth in Rip1Tag2 transgenic mice. Mice were stratified (in a 1:1 ratio) according to sex and age, and littermates were balanced between the nintedanib and the vehicle-treated control group and then treated with 50 mg/kg nintedanib daily per os (p.o.) beginning at the age of 9 weeks for 3 weeks, a time period in which tumor angiogenesis and tumor growth are highly active (26). Nintedanib-treated mice showed a strong reduction in the total tumor burden per mouse, as well as less macroscopically detectable tumors (Fig. 1A and B). In addition, the few macroscopically detectable tumors in nintedanib-treated animals were white, in contrast with the red, highly vascularized appearance of the tumors in vehicle-treated control animals, indicating decreased vascularization (Fig. 1C). Next, we assessed whether this dramatic reduction of primary tumor growth translated into a prolongation of survival of Rip1Tag2, as it has been observed for sunitinib with this mouse model (22). Treatment with nintedanib (50 mg/kg, 1×/d, p.o.) or placebo was initiated at the age of 10 weeks and continued open end. Animals were euthanized before suffering of hypoglycemia, by fulfilling the termination criteria as described in Materials and Methods. We observed a significant (log-rank test, P < 0.001) increase in the survival of the nintedanib-treated group (median survival, 55 days on treatment) as compared with the vehicle-treated control group (median survival, 24 days on treatment; Fig. 1D). Interestingly, tumors of nintedanib-treated animals displayed a white color even at the terminal stage (data not shown). Together, these results demonstrate an efficient antitumor effect of nintedanib in Rip1Tag2 mice by reducing primary tumor burden and significantly extending survival time.

Reduced tumor blood vessel density correlates with increased tumor cell apoptosis

To determine the antiangiogenic capabilities of nintedanib, we quantified microvessel density (MVD) by IF staining with the endothelial cell marker CD31 on 3-week nintedanib and placebo-treated tumors. Nintedanib treatment significantly reduced MVD by more than 50% (Fig. 2A). Most antiangiogenic drugs are known to mediate their anti-tumor activity by increasing apoptosis rather than inhibiting proliferation (26). Consistently, cleaved Caspase 3 (cCasp3) IF
staining revealed that the strong reduction of MVD was accompanied by an increase of tumor cell apoptosis in nintedanib-treated tumors as compared with controls (Fig. 2B). This finding is supported by the increased levels of double-strand breaks as detected by TUNEL assay (Supplementary Fig. S1B) and the lack of a change in tumor cell proliferation as determined by phospho-histone-3 staining (pH3; Supplementary Fig. S1C). These data suggest that the antitumor effect of nintedanib is mainly caused by increased tumor cell apoptosis triggered by the strong reduction of MVD. A direct effect of nintedanib on tumor cells is rather unlikely, since treatment of cultured insulinoma cells derived from Rip1Tag2 mice only reduced tumor cell numbers at high nintedanib concentrations ($IC_{50} = 1.891 \mu mol/L$)—levels that are not reached when treating mice daily with 50 mg/kg (ref. 11; Supplementary Fig. S1D).

Nintedanib-treated tumor blood vessels display a mature phenotype
Nintedanib not only inhibits signaling of VEGF receptors and FGF receptors, both important receptor families for vascular sprouting and therefore neovessel formation, but also targets PDGF receptors (11). Endothelial cell-derived PDGF-BB attracts perivascular cells by binding to PDGFRβ expressed by them, and perivascular cells subsequently cover the abluminal surface of the vessel tube to mediate vessel stability and functionality (27). Interestingly, the targeting of the perivascular cell coverage in addition to inhibiting endothelial cell sprouting has led to a

Figure 1.
Reduced tumor volume and improved survival upon nintedanib treatment of Rip1Tag2 transgenic mice. A and B, shown are total tumor volumes (A) and number of macroscopically detectable tumors (B) per Rip1Tag2 mouse treated for 3 weeks with nintedanib or vehicle control starting at the age of 9 weeks (pooled analysis of 3 independent experiments). Vehicle: n = 15 mice; nintedanib: n = 20 mice. Statistical analysis by Mann–Whitney U test; ***; P < 0.001. C, tumor nodules (arrows) in the pancreas of nintedanib-treated mice are barely detectable, because they are less frequent and of whitish color as compared with more frequent and red-colored tumors in vehicle-treated Rip1Tag2 mice. Scale bar, 2 mm. D, survival trial of nintedanib treatment starting at the age of 10 weeks and 2 days. Median survival: vehicle group: 24 days on treatment; nintedanib group: 55 days on treatment. Vehicle: n = 16 mice; nintedanib: n = 15 mice. Log-rank test; P < 0.001.
beneficial antitumor effect (21). We thus examined the phenotype of the tumor blood vessels present after 3 weeks of nintedanib treatment. Perivascular cell coverage was assessed by staining for the perivascular cell marker neuron-glial antigen 2 (NG2; ref. 27). Quantification of NG2 staining revealed that nintedanib strongly reduced the total number of NG2+ perivascular cells per field of view (Fig. 3A; Supplementary Fig. S2A). Moreover, while in vehicle-treated control tumors almost all perivascular cells found attached to vessels, with the loss of MVD upon nintedanib treatment, an increased fraction of perivascular cells is found without contact to vessels (Fig. 3A; Supplementary Fig. S2B). Still, the few blood vessels in nintedanib-treated tumors appeared to be substantially covered by perivascular cells and displayed a mature phenotype (Fig. 3A and B). We thus tested the functionality of the treatment-resisting blood vessels by intravenously injecting fluorescein-labeled Lectin shortly before sacrificing the animals. While in the vehicle-treated tumors only a minority of blood vessels was nonfunctional (~8%), nintedanib treatment further reduced the amount of nonfunctional vessels (~4%; Fig. 3C).

One important hallmark of the aberrant phenotype of tumor blood vessels is the increased leakiness, leading to increased interstitial fluid pressure and thus reduced intratumoral delivery of chemotherapeutic agents (28, 29). We have assessed blood vessel leakiness by intravenously injecting fluorescein-labeled Dextran (70 kDa) into vehicle and nintedanib-treated animals. Leaky spots, i.e., fluorescein-labeled Dextran in the abluminal compartment of blood vessels identified by CD31 staining, were mainly detected at the tumor border. The quantification of leaky spots at the tumor border did not reveal a significant difference between vehicle and nintedanib-treated mice (data not shown). Interestingly though, nintedanib treatment reduced the amount of leaky spots in the tumor center (Fig. 3D). Finally, we determined the consequences of the strong reduction in MVD observed in nintedanib-treated tumors on tumor oxygenation by pimonidazole staining. Three weeks of nintedanib treatment significantly increased the number of tumors with hypoxic areas and the hypoxic area fraction of hypoxic tumors (Supplementary Fig. S3A–S3C). However, approximately 85% of nintedanib-treated tumors did not display any signs of hypoxia, further indicating that nintedanib-resistant vessels are mature and fully functional. Tumor hypoxia has often been causally linked with the induction of local tumor invasiveness (22). Interestingly therefore, hypoxia in nintedanib-treated insulinoma was often found close to clearly noninvasive tumor borders (Supplementary Fig. S3D).

In summary, nintedanib treatment leads to a strong reduction in the total amount of NG2-positive perivascular cells. Importantly, the blood vessels that survive nintedanib treatment are patent, and, thus, the strong reduction in MVD causes hypoxia only in a minority of tumors.

**Tumor lymphangiogenesis is not affected by nintedanib treatment**

*In vivo* kinase assays have shown that nintedanib inhibits VEGF receptor 3 at a concentration comparable with the concentration needed to inhibit VEGF receptor 2 signaling (10). Although VEGF receptor 3 is expressed on blood vessel tip cells with substantial functions in blood vessel sprouting (30), its classical role is attributed to mediating lymphangiogenesis. Since the expression of VEGF-C, the cognate ligand of VEGF receptor 3, in tumor cells of Rip1Tag2;Rip1VEGF-C double-transgenic mice leads to increased tumor lymphangiogenesis and facilitates lymphogenic metastatic spreading, the inhibition of tumor lymphangiogenesis may represent an interesting therapeutic opportunity (31). On the other hand, Sennino and colleagues reported that antiangiogenic treatments of Rip1Tag2 mice increased tumor lymphangiogenesis and lymphatic metastasis (32).

To assess whether nintedanib affects tumor lymphangiogenesis in Rip1Tag2 transgenic mice, we first analyzed peritumoral lymphatic coverage of tumors of Rip1Tag2 mice treated with

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**Figure 2.**

Nintedanib reduces microvessel density and induces apoptosis. A and B, microvessel density (A) and apoptosis rate (B) of 3-week nintedanib-treated Rip1Tag2 tumors starting from 9 weeks of age were assessed by immunofluorescence staining for CD31 (green) and cCasp3 (red), respectively. Representative immunofluorescence pictures (×20 magnification) are shown. Values were obtained by counting the number of vessels or apoptotic cells per area of each microscopic field of view. Bar graphs display mean ± SEM, and statistical analysis was performed using an unpaired Student t test. Vehicle: n = 4 mice; nintedanib: n = 7 mice. **, P < 0.01; ***, P < 0.001. Scale bars, 100 μm.
Figure 3.

Blood vessels resisting nintedanib treatment display a mature phenotype and retain their function. A, Rip1Tag2 mice were treated with nintedanib for 3 weeks starting at 9 weeks of age. Representative images (×20 magnification) of an immunofluorescence staining of pancreatic sections for tumor blood vessels (CD31, green), perivascular cells (NG2, red), and cell nuclei (DAPI, blue) are shown. Scale bars, 100 μm. B, quantification and analysis of the relative localization of NG2⁺ perivascular cells to CD31⁺ blood vessels revealed in nintedanib-treated tumors a slightly reduced NG2⁺ perivascular cell coverage of the remaining blood vessels. The percentage of perivascular cell-covered blood vessels is displayed per each field of view. Vehicle: n = 4 mice; nintedanib: n = 7 mice. Scale bars, 100 μm. C, blood vessel patency was assessed by i.v. injection of fluorescein-labeled Lectin (green) and immunofluorescence costaining for CD31 (red) and cell nuclei (DAPI, blue). CD31⁺ blood vessels without signs of Lectin signal were compared with the total number of vessels per field of view and displayed as mean ± SEM. Representative images (×20 magnification) are shown as single channels in gray scale and merged. Arrowheads point toward blood vessels without perfusion. Scale bars, 100 μm. D, blood vessel leakiness was analyzed by injecting fluorescein-labeled Dextran (70 kDa) i.v. Quantification and representative immunofluorescence images of fluorescein-labeled Dextran (70 kDa; green) and CD31⁺ blood vessels (red) are shown. Cell nuclei are visualized by DAPI staining (blue). The bar graph indicates the number of Dextran-positive intratumoral leaky spots per tumor area. Data are displayed as mean ± SEM per field of view. Arrows point toward intratumoral leaky spots and arrowheads toward leaky spots along the tumor border (quantification not shown). Vehicle: n = 6 mice; nintedanib: n = 7 mice. Scale bar, 100 μm. Statistical analysis was performed using an unpaired Student t test (B–D); *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Nintedanib Therapy for PNET

Figure 4.
Tumor lymphangiogenesis is not affected by nintedanib treatment. A and B, the percentage of LYVE-1-positive lymphatic endothelium (red, arrowheads) covering the perimeter of insulin-positive β-cell tumors (insulin, green) stained by immunofluorescence was quantified in Rip1Tag2 (A) and Rip1Tag2;Rip1-VEGF-C (B) mice treated for 3 weeks with nintedanib or vehicle control starting at 9 weeks of age. DAPI staining visualizes cell nuclei (blue). Representative microphotographs are shown (×10 magnification). Bar graphs represent mean lymphatic tumor coverage per tumor ± SEM. Statistical analysis was performed using an unpaired Student t test. A, vehicle: n = 4 mice; nintedanib: n = 7 mice; B, n = 3 mice for each group. Scale bars, 100 μm.

Nintedanib does not induce tumor invasiveness and metastasis

Recent reports have suggested that antiangiogenic substances, such as sunitinib, can increase local invasiveness and lymph node and liver metastasis in Rip1Tag2 transgenic mice and other mouse models of cancer, raising major concerns about the use of antiangiogenic therapies in patients (7, 22, 33). We therefore analyzed the effect of different nintedanib treatment regimens on local invasiveness and distant metastasis by histologic grading of hematoxylin and eosin–stained pancreas sections (see Material and Methods and Supplementary Fig. S1A; ref. 25). In the 3-week nintedanib treatment regimen initiated at 9 weeks of age, we could neither detect a significant increase in the percentage of microinvasive lesions (IC1) nor an increase in macroinvasive lesions (IC2; Fig. 5A). In addition, this treatment regimen did not lead to an increase in local lymph node and liver metastases as detected by staining for SV40 T antigen, the oncogene expressed by β-tumor cells in Rip1Tag2 transgenic mice (Fig. 5B and C). Of note, in all of the mice analyzed, liver metastases exceeding 10 cells per cross section were rarely observed and were restricted to a few mice (data not shown). To rule out a transient and reversible increase of local tumor invasiveness, we initiated treatment at around 9 weeks of age and analyzed pancreata after 5 days of nintedanib treatment. This treatment regimen was sufficient to reduce MVD and tumor volume at borderline significance, yet it also did not increase local tumor invasiveness (Supplementary Fig. S4A–S4C).

A meaningful assessment of a potential prometastatic effect of compounds prolonging overall survival in clinical trials is often hampered by the latency of metastasizing tumor cells. We therefore asked whether the survival benefit achieved by nintedanib treatment would be paralleled by increased liver metastasis when analyzing vehicle and nintedanib-treated groups at their endpoints. Although nintedanib treatment increased survival by more than 4 weeks, the number of liver metastases per mouse was not increased compared with vehicle-treated mice (Supplementary Fig. S5).

Since treatment with nintedanib for 3 weeks was initiated at an intermediate stage of tumorigenesis, the possibility remained that nintedanib treatment initiated at late stage of Rip1Tag2 tumor-growth may instead increase local invasiveness. However, treatment with nintedanib for 5 days initiated at a late tumor stage (11 weeks of age) did not induce local invasiveness, whereas a substantial reduction in MVD with a significant reduction in tumor volume was observed (Fig. 5D; Supplementary Fig. S6A and S6B).

Sunitinib does not induce tumor invasion and metastasis

Although several publications have shown increased invasive-ness and metastasis upon antiangiogenic treatments in the Rip1-Tag2 mouse model of PNET (7, 22, 33), this issue remains controversial, and solid data reporting similar findings in human clinical trials are lacking (34). In particular, it is not known whether the proinvasive phenotype observed in some preclinical cancer models can be attributed to inhibition of the VEGF-A/VEGFR-2 axis alone, and is therefore applicable to all VEGF...
pathway inhibitors or whether it is a consequence of increased tumor hypoxia caused by multitarget tyrosine kinase inhibitors, such as sunitinib (7, 22, 33, 35).

To specifically address this question, we analyzed the local invasiveness of Rip1Tag2 tumors treated with the antiangiogenic small-molecule tyrosine kinase inhibitor sunitinib, which has previously shown to be especially efficient in enhancing tumor invasiveness and metastasis in the Rip1Tag2 mouse model (22, 36). Interestingly, although a 3-week sunitinib treatment effectively reduced MVD and primary tumor volumes compared with control-treated mice, but did not affect tumor invasiveness (Supplementary Fig. S4A–S4C).

Blocking VEGFR 1-3 signaling does not induce local tumor invasiveness

To further characterize the general response patterns to different antiangiogenic TKI in Rip1Tag2 mice, we performed therapy studies with PTK787/ZK222584 (PTK/ZK), which mainly inhibits VEGF receptor 1-3 and PDGF receptor signaling.

### Figure 5.
Nintedanib and sunitinib do not induce tumor invasiveness and metastasis.

A, tumors of Rip1Tag2 mice treated for 3 weeks with nintedanib or sunitinib in two separate experiments starting at 9 weeks of age were classified into any of the 3 categories as indicated by the percentages of the tumors inside the bars and the numbers of total tumors per experimental group, which are displayed on top of the bars. Vehicle: n = 6 mice; nintedanib: n = 9 mice, data of two independent experiments were pooled. Control: n = 10 mice; sunitinib: n = 11 mice. Statistical analysis was performed using the Fisher exact test; *, P < 0.05.

B, pancreatic lymph node metastases were analyzed in 23 vehicle-treated (10 mice) and 48 nintedanib-treated (14 mice), and in 4 control-treated (10 mice) and 14 sunitinib-treated (11 mice) lymph nodes. Fisher exact test; P = 0.6252 (nintedanib treatment), P = 1 (sunitinib treatment). C, metastasis to the liver was analyzed on 9 histologic liver sections per mouse of vehicle-treated (4 mice) and nintedanib-treated (7 mice), and in control-treated (10 mice) and sunitinib-treated (11 mice) mice. Mann–Whitney U test. D, grading of tumor stages in Rip1Tag2 mice treated for 5 days with nintedanib or vehicle control initiated at 11 weeks of age. Vehicle: n = 16 mice; nintedanib: n = 18 mice; pooled data of 3 independent experiments are shown.
We treated Rip1Tag2 mice starting from 9 weeks of age with PTK/ZK (100 mg/kg) for 3, 4, 5, or 6 weeks, whereas the control treatment group was treated for 3 weeks only, to avoid preterm death. Prolonged PTK/ZK treatment efficiently reduced MVD and had varying effects on tumor volumes (Supplementary Fig. S7A and S7B). Interestingly, we did not observe revascularization even in the 5- and 6-week treatment cohorts. None of the PTK/ZK treatment groups displayed any sign of increase local tumor invasiveness compared with the control cohort, even though the prolonged treatment provided additional time for the tumors to progress (Fig. 6A). On the contrary, 3- and 6-week PTK/ZK treatment rather reduced the percentage of macroinvasive (IC2) lesions. Similar to the nintedanib treatment, we assessed whether 5 days of PTK/ZK treatment initiated at late tumor stage (11 weeks of age) would increase tumor invasiveness. Although we could not detect any difference in the percentage of macroinvasive tumors between the PTK/ZK and the control-treated cohort, there was a shift toward an increased percentage of microinvasive lesions in PTK/ZK-treated mice (Fig. 6B). To further follow up and validate these results, we analyzed the entrapment of amylase-positive cells of the exocrine pancreas within insulin-positive tumors by immunofluorescence staining as has been previously described (33). Tumors entrapping amylase-positive cells are
an indicator of macroinvasiveness (Fig. 6C). In concordance with the histologic grading, 5-day PTK/ZK treatment initiated at late tumor stage (11 weeks of age) also did not significantly alter the percentage of tumors entrapping cells of the exocrine pancreas, despite strongly reducing MVD and tumor volume (Fig. 6C, Supplementary Fig. S7C and S7D). PTK/ZK treatment initiated at an early tumor stage (9 weeks of age) for 5 days rather reduced local invasiveness (Supplementary Fig. S4C). In addition, treating Rip1Tag2;Rip1-VEGF-C mice with PTK/ZK for 3 weeks also did not increase local tumor invasiveness (Fig. 6D).

Based on these data, we conclude that targeting tumor angiogenesis in Rip1Tag2 transgenic mice by a variety of therapy regimens with the multikinase inhibitors nintedanib, sunitinib, or PTK/ZK efficiently represses tumor angiogenesis, but does not increase the incidence of invasive tumors or metastasis.

Discussion

Based on numerous promising results from preclinical cancer models, the strategy of targeting tumor blood vessels and thus reducing the amount of oxygen and nutrients available to tumors has been translated with great enthusiasm into clinical practice (37). In clinical trials and routine therapy, most antiangiogenic substances have increased progression-free survival, yet they have failed to substantially prolong overall survival in a variety of solid tumor types (5, 38). As an exception, treatment of patients with advanced PNET with the antiangiogenic TKI sunitinib has raised the possibility that this group of cancer is particularly sensitive to antiangiogenic therapy (3). At the same time, this clinical trial reproduced the beneficial effect observed in preclinical experiments targeting both endothelial- and perivascular cells with sunitinib in the Rip1Tag2 mouse model of PNET (21, 22). These and other data have proven the Rip1Tag2 model highly predictive in translating findings from the bench to the bedside (20, 39, 40).

In the present study, we have performed an in-depth evaluation of the broad-spectrum antiangiogenic small-molecule TKI nintedanib in the Rip1Tag2 preclinical mouse model of PNET. Nintedanib, which mainly inhibited VEGF, PDGF, and FGF tyrosine kinase receptors and Src nonreceptor tyrosine kinase (10), exerted a strong antiangiogenic effect in the Rip1Tag2 transgenic mouse model of neuroendocrine carcinoma of the pancreas, which resulted in reduced tumor volumes and increased animal survival. Nintedanib extensively reduced microvessel density and tumor volume. Interestingly though, despite nintedanib’s inhibitory action on perivascular cells, the blood vessels that remained were mature, tightly covered by perivascular cells, well perfused, and showed reduced intratumoral leakiness. Although the vascularity in the placebo-treated group was already well perfused and only rarely showed signs of intratumoral leakiness, prolonged (3 week) nintedanib treatment led to further blood vessel normalization with only a marginal increase of tumor hypoxia. Since blood vessel normalization is associated with enhanced delivery of chemotherapeutic agents into tumors (28, 29), nintedanib should not only be further evaluated for its own anticancer effect but also for a potential synergistic function by enhancing the intratumoral delivery of anticancer agents. Nintedanib has been shown well tolerated in the treatment of idiopathic pulmonary fibrosis and cancer patients (13, 14) and, hence, it should be clinically tested on patients with PNET and other angiogenic cancer types in combination with conventional chemotherapy. In addition, nintedanib could be used when tumors become refractory to therapies targeting mainly the VEGF-A/VEGFR2 axis by FGF signaling-mediated revascularization—as it has been previously shown for brivanib in the Rip1Tag2 mouse model (41). Brivanib is an antiangiogenic TKI displaying a similar target spectrum as nintedanib; both compounds are inhibiting FGFR signaling in addition to VEGFR and PDGF signaling.

Surprisingly, nintedanib did not affect tumor lymphangiogenesis in Rip1Tag2 mice as well as in Rip1Tag2 mice in which tumor lymphangiogenesis was induced by tumor cell–specific expression of VEGF-C (Rip1Tag2;Rip1-VEGF-C double-transgenic mice). In concordance with these findings, we have previously shown that the antiangiogenic TKI PTK/ZK, mainly blocking VEGFRs 1-3, was also not able to inhibit established and ongoing tumor lymphangiogenesis in the Rip1Tag2;Rip1-VEGF-C model despite a substantial reduction of VEGFR3 phosphorylation (26). These data indicate that VEGF-C–induced tumor lymphangiogenesis may eventually rely on factors other than VEGF-C and that pathways not targeted by nintedanib and PTK/ZK are at play. These observations warrant further investigations.

Previous work has raised concerns that antiangiogenic therapy might increase tumor invasiveness and distant metastasis in the Rip1Tag2 model (7, 22, 33, 36, 42). Some work attributed the invasiveness-promoting effect to a general feature of antiangiogenic drugs (22, 33), presumably by inducing a hypoxia-driven epithelial–mesenchymal transition. In contrast and consistent with our findings, a recent report shows that nintedanib not only repressed primary tumor growth of xenotransplanted NSCLC and exocrine pancreas carcinoma by reducing vessel density, maturation, and perfusion but also repressed metastatic dissemination (43). Moreover, others have observed increased tumor aggressiveness only with drugs that in addition to the VEGF-A/VEGFR2 axis also targeted perivascular cells, such as sunitinib (35). To contribute to this important discussion, we not only analyzed potential changes in invasiveness induced by the broad spectrum TKI nintedanib and sunitinib, but also when angiogenesis was inhibited by mainly targeting VEGF and PDGF receptors with PTK/ZK. In our Rip1Tag2 mice, nintedanib, sunitinib, and PTK/ZK did not substantially increase local invasiveness, which stands in contrast with what has been reported by others (7, 22, 33, 35, 36, 42). Supporting our findings is the fact that in cancer patients solid data are lacking that show an increased invasiveness and metastasis induced by antiangiogenic therapies, possibly with the exception of glioblastoma multiforme (44). In addition, it has been shown that sunitinib treatment of metastatic renal cell carcinoma did not adversely alter the patients’ clinical outcome (34). An alternative explanation for this apparent discrepancy has been discussed previously (35): Rip1Tag2 mice have been bred in isolation between different laboratories for approximately two decades which may have led to genetic drifts resulting in altered susceptibilities to the induction of tumor invasion by antiangiogenic therapies. The delineation of the mechanisms driving this discrepancy will be part of exciting future research which may lead to the discovery of novel factors and pathways determining the susceptibility to cancer metastasis, induced by changes in the tumor microenvironment.

In summary, our preclinical data together with the previous reports of successful clinical applications in patients strongly encourage the evaluation of nintedanib treatment as a novel therapeutic strategy in PNET patients with advanced disease.
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G. Christofori reports receiving commercial research grants from Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: R. Bill, A. Zumsteg, F. Hilberg, G. Christofori
Development of methodology: R. Bill, A. Zumsteg, D. Johansson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Bill, E. Fagiani, A. Zumsteg, H. Antoniadis, D. Johansson, I. Albrecht, G. Christofori
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Bill, E. Fagiani, A. Zumsteg, H. Antoniadis, D. Johansson, S. Haefliger, I. Albrecht, G. Christofori
Writing, review, and/or revision of the manuscript: R. Bill, E. Fagiani, A. Zumsteg, F. Hilberg, G. Christofori
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Bill, F. Hilberg, G. Christofori
Study supervision: E. Fagiani, G. Christofori

References

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Nintedanib Therapy for PNET

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Ruben Bill, Ernesta Fagiani, Adrian Zumsteg, et al.

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