Vascular Targeting in Pancreatic Cancer: The Novel Tubulin-Binding Agent ZD6126 Reveals Antitumor Activity in Primary and Metastatic Tumor Models

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Abstract

ZD6126 is a novel vascular-targeting agent that acts by disrupting the tubulin cytoskeleton of an immature tumor endothelium, leading to an occlusion of tumor blood vessels and a subsequent tumor necrosis. We wanted to evaluate ZD6126 in primary and metastatic tumor models of human pancreatic cancer. Nude mice were injected orthotopically with L3.6pl pancreatic cancer cells. In single and multiple dosing experiments, mice received ZD6126, gemcitabine, a combination of both agents, or no treatment. For the induction of metastatic disease, additional groups of mice were injected with L3.6pl cells into the spleen. Twenty-four hours after a single-dose treatment, ZD6126 therapy led to an extensive central tumor necrosis, which was not seen after gemcitabine treatment. Multiple dosing of ZD6126 resulted in a significant growth inhibition of primary tumors and a marked reduction of spontaneous liver and lymph node metastases. Experimental metastatic disease could be significantly controlled by a combination of ZD6126 and gemcitabine, as shown by a reduction of the number and size of established liver metastases. As shown by additional in vitro and in vivo experiments, possible mechanisms involve antivascular activities and subsequent antiproliferative and proapoptotic effects of ZD6126 on tumor cells, whereas direct activities against tumor cells seem unlikely. These data highlight the antitumor and anti-metastatic effects of ZD6126 in human pancreatic cancer and reveal benefits of adding ZD6126 to standard gemcitabine therapy.

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Keywords: vascular targeting, pancreatic cancer, cytoskeleton, orthotopic tumor model, dorsal skinfold chamber.

Introduction

The importance of vascular network in tumor growth and metastasis is now widely recognized and the ability to interfere with this aspect of tumor survival offers a significant therapeutic potential. Two key approaches have been explored to specifically inhibit tumor blood supply [1,2]. The first approach includes preventing the development of new tumor blood vessels (angiogenesis) by using antiangiogenic agents, thereby limiting tumor growth [2]. The second approach tries to destroy existing abnormal tumor vessels by using specific antivascular agents (vascular targeting) [3]. Vascular-targeting drugs act largely by exploiting the structural and physiological differences between normal and pathologic vasculature to selectively disrupt and occlude immature tumor vessels. As a large number of tumor cells depend on a relatively small number of blood vessels, vascular targeting results in significant tumor hypoxia and cell death, producing widespread tumor necrosis [4,5].
action differs from other tubulin-binding agents, such as colchicine and vinca alkaloids, as these agents are thought to act primarily through a direct cytotoxic effect brought about by mitotic arrest [8,9]. In different tumor xenograft models, ZD6126 treatment has been shown recently to induce a selective disruption of tumor blood vessels and an extensive central tumor necrosis [10–15]. Dose–response studies showed that these effects were seen at doses that were at least 8- to 16-fold lower than the maximum tolerated dose (MTD), demonstrating a wide therapeutic margin in those animal models [10]. Despite the potent antivascular effects of ZD6126 in the center of tumors, a thin rim of viable tumor tissue is found at the periphery of tumors, where adjacent normal vessels could still provide nutrients and oxygen to peripheral tumor cells. These cells might rapidly reestablish a new tumor mass. However, due to the high proliferation rate of those cells, they might be still good targets for conventional chemotherapy.

Because most cancer deaths in humans are due to consequences of metastatic disease, it is important that anticancer agents and combinations of drugs are not only active in primary tumors but also prevent metastatic spread or show efficacy in established metastatic disease. Pancreatic cancer, in particular, remains an unsolved health problem, with an overall 5-year survival rate of only 1% to 4% due to an inability to detect this disease at early stages. Most of the patients present with liver or lymph node metastases at the time of diagnosis [16]. The aim of this study was to assess the benefits of combining the new vascular-targeting agent ZD6126 and the standard pyrimidine antimetabolite gemcitabine in primary and metastatic human pancreatic cancer. Orthotopic and heterotopic primary tumor models and a model of established liver metastases were chosen to study the efficacy of this combination therapy. Immunohistochemistry (IHC), in vivo videomicroscopy (IVM), and in vitro proliferation assays were used to determine the effects of ZD6126 on endothelial cells and human L3.6pl pancreatic cancer cells [17].

Materials and Methods

**Cell Lines and Culture Conditions**

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured, as described before [18], and used between the third and fifth passage. The highly proliferative human pancreatic cancer cell line L3.6pl [17] was maintained in Dulbecco’s minimal essential medium, supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, a two-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and a penicillin–streptomycin mixture (Flow Laboratories, Rockville, MD), to constitute a complete Dulbecco’s minimal essential medium. Monolayers were maintained on plastic and incubated in 5% CO₂ and 95% oxygen at 37°C. The culture was free of *Mycoplasma* and pathogenic murine viruses and was maintained for no longer than 12 weeks.

**Animals and Compounds**

Male athymic nude mice (Balb/c nu/nu) were purchased from Charles River Laboratories (Sulzfeld, Germany, Indianapolis, IN). Animals were housed and maintained in laminar flow cabinets under specific pathogen-free conditions at the University of Regensburg (Regensburg, Germany) and used in accordance with institutional and governmental guidelines at 8 to 12 weeks of age.

ZD6126 was kindly provided by AstraZeneca and dissolved in a solution of 0.05% sodium carbonate in physiological saline. Gemcitabine (Gemzar; Eli Lilly and Co., Indianapolis, IN) was obtained commercially. Antibodies and reagents as follows were purchased: rat antimouse CD31/PECAM-1 (Pharhingen, San Diego, CA); peroxidase-conjugated goat antirat IgG [H+L] (Jackson Immuno Research Laboratories, West Grove, CA); streptavidin–biotin detection system (Dako A/S, Glostrup, Denmark); stable 3,3′-diaminobenzidine (DAB; Research Genetics, Huntsville, AL); 3-amino-9-ethylcarbazole (Biogenex Laboratories, San Ramon, CA); Gill’s hematoxylin (Sigma Chemical Co., St. Louis, MO); Ki67 (Dako A/S); an apoptosis detection kit for the terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL) procedure (Promega, Madison, WI); and an MTT cell viability assay kit for *in vitro* proliferation assays (R&D Systems, Minneapolis, MN). Other consumables used were as follows: OCT compound (Miles, Inc., Elkhart, IN); positively charged Superfrost slides (Fisher Scientific Co., Houston, TX); and ProLong solution (Molecular Probes, Eugene, OR).

**Cell Proliferation Assay**

For an assessment of ZD6126 activity directed against human pancreatic cancer cells and cultured endothelial cells, we used an *in vitro* MTT cell viability assay, which was conducted on cultured L3.6pl cells and HUVECs, as described before [19,20]. Briefly, $15 \times 10^3$ cells/well were plated in 96-well plates in complete medium. After 24 hours of attachment, the active compound ZD6126 phenol was added in different concentrations (0.001–1000 μM), and the plates were incubated for another 48 hours. Cells were washed with phosphate-buffered saline (PBS), the MTT reagent was added according to the manufacturer’s recommendations, and absorbance was measured at 570 nm. The IC<sub>50</sub> was defined as the drug concentration that causes a 50% inhibition of cell proliferation. All experiments were replicated three times.
Dose–Response Assays for Gemcitabine

Doses of ZD6126 and gemcitabine were chosen based on the results of previous reports [10,13,21] and our own preliminary experiments, respectively. In vitro, we confirmed the efficacy of gemcitabine as a chemotherapeutic agent against L3.6pl pancreatic cancer cells using the MTT cell viability assay, as described before [20]. The IC_{50} value for gemcitabine was 0.0487 μg/ml in this assay, demonstrating a high sensitivity of L3.6pl cells. In contrast, AsPC human pancreatic cancer cells showed IC_{50} values of 452.6 μg/ml for gemcitabine. Moreover, in vivo dose–response experiments were performed to evaluate the therapeutic effect of gemcitabine on our animal models. Five mice per group were treated biweekly with 500, 250, 125, 62, 31, 15.5, and 7.5 mg/kg gemcitabine by intraperitoneal (i.p.) administration, resulting in a growth inhibition of orthotopic L3.6pl tumors by 65%, 58%, 55%, 46%, 36%, 31%, and 0.2%, respectively. Hence, doses of 100 mg/kg gemcitabine were used for subsequent experiments.

Single-Dose Therapy

For an evaluation of a single-dose therapy in established primary pancreatic tumors, 24 mice were injected orthotopically with 1 × 10^6 viable human L3.6pl cells suspended in 0.2 ml of PBS on day 0, as described previously [17,22]. After 14 days, when primary tumors were established (tumor volume 125–512 mm³), animals received a single dose of ZD6126 (150 mg/kg, i.p.; n = 6), a single dose of gemcitabine (100 mg/kg, i.p.; n = 6), a combination of both agents at the same doses (n = 6), or no treatment (n = 6). Animals were sacrificed 24 hours after single-dose treatments, and tumors were removed for histomorphologic examination (hematoxylin and eosin [H&E] staining).

Primary Tumor Model (Chronic Therapy)

For the assessment of tumor growth inhibition and metastatic prevention through repeated dosage, another 40 mice were injected orthotopically with 1 × 10^6 L3.6pl cells on day 0 [17]. On day 9, four mice were sacrificed, and primary tumors with volumes of 64 to 216 mm³ but without metastases were found, suggesting that, at that time, medical treatment would be directed against well-established but organ-confined pancreatic tumors and may prevent metastatic disease. The treatment of the remaining animals was started on the same day. Four groups of animals received either ZD6126 (75 mg/kg, i.p., daily (n = 8); or gemcitabine twice weekly (100 mg/kg, i.p.; n = 10); or a combination of both regimens (n = 8); or no treatment (n = 10). All mice were sacrificed on day 25, as the control animals became moribund. Pancreatic tumors were removed and weighed, and five tumors per treatment group were chosen randomly for further analysis. One part of each of those tumors was fixed in formalin and embedded in paraffin for immunohistochemical evaluation. The other parts of the primary tumors were prepared for frozen sectioning. Liver and lymph node metastases as well as peritoneal carcinosis were assessed macroscopically and confirmed histologically by H&E staining. Tumor nodules ≥1 mm in diameter were counted on the entire liver surface.

Liver Metastasis Model (Chronic Therapy)

To evaluate the efficacy of palliative treatment in metastatic disease, a liver metastasis model was used. For the production of experimental liver metastases, 1 × 10^6 L3.6pl cells were injected heterotopically into the spleen of another 40 nude mice. After 14 days, four mice were sacrificed, and all of them showed extended macroscopic liver metastases. Thus, the treatment of the remaining animals was started on day 14 with either ZD6126 (n = 9) or gemcitabine (n = 9), or a combination of both agents (n = 9), or no treatment (n = 9), using the same dosages as described in the primary tumor model. Finally, mice were sacrificed 29 days after heterotopic tumor cell injection, as the control animals became moribund. The liver and the spleen were removed and weighed, and liver metastases were counted on the entire liver surface, measured, and confirmed by H&E staining.

Immunohistochemical Analyses

Immunohistochemical analyses of tumor vascularization in primary tumors were performed on frozen sections from the primary tumor model. Sections measuring 8 to 9 μm were air-dried, fixed in cold acetone for 5 minutes, fixed in acetone/chloroform for 5 minutes, fixed in acetone for 5 minutes, and then washed in PBS. After that, CD31 immunohistochemical staining was performed, as described previously, and microvessel density was assessed [23]. The evaluation of tumor cell proliferation was performed using a Ki67 monoclonal antibody. Therefore, paraffin-embedded specimens of primary tumors were used, and 4- to 6-μm sections were prepared on slides, dried overnight, deparaffinized in xylene, and rehydrated in 100%, 95%, and 80% ethanol and PBS. Ki67 “antigen retrieval” was achieved by microwaving sections for 5 minutes. Then IHC was performed using a standard streptavidin–biotin method with DAB as chromogen. Sections were analyzed microscopically, and the numbers of positive staining cells were counted. Paraffin-embedded tissues were also analyzed for evidence of apoptosis by TUNEL fluorescein isothiocyanate (FITC) staining using a commercial apoptosis detection kit. For all immunohistochemical studies, five sections per tumor and five randomly chosen tumors per treatment group were analyzed (25 sections per group). Microscopic assessment was performed by two independent observers who were blinded to all experimental data, through an analysis of 6 to 10 high-power fields (hpf) per section (hpf, 0.159 mm²).

IVM of Neovascularization

Tumor neovascularization in vivo was analyzed using a window model, as described previously [24–26]. Briefly, mice were implanted subcutaneously with 1 × 10^6 L3.6pl cells in a transparent dorsal skinfold chamber on day 0. From day 1, animals received ZD6126, 75 mg/kg, i.p., daily (n = 5)
for a maximum of 10 days, or received no treatment (n = 5).
Tumor neovessels were assessed by a blinded observer
on days 2, 3, 5, 7, and 8 using IVM (Zeiss Axiotech Vario
microscope, Göttingen, Germany). Then, average microvessel
density (combined vessel length [cm] / tumor area [cm²]) was
calculated out of 8 to 12 single measurements using an Image
J software (Wayne Rasband, Version 1.25s; NIH, Bethesda,
MD) by generating horizontal grid lines for every 50 pixels.
Tumor vessels crossing the grid lines were individually mea-
sured, whereas vertically aligned vessels were not included
in the analysis. A conversion factor of 0.72 m/pixel was used
to calculate the actual vessel diameter.

Statistical Analysis
Pancreatic tumor weight, body weight, in vitro cell pro-
liferation (MTT assay), IHC quantification of CD31, Ki67 and
TUNEL, and microvessel density measured by IVM were com-
pared using one-way ANOVA with a Student–Newman–Keuls
multiple comparisons test (InStat 3.0 Statistical Software;
Graphpad Software, San Diego, CA). The relative rates of
liver and lymph node metastases within groups were com-
pared by Fisher’s exact test. All analyses were performed
with P < .05 considered as significant.

Results
Cell Proliferation Assay
A significant antiproliferative activity of ZD6126 phenol
on HUVECs could be already observed at concentrations of
0.1 μM (IC50 = 100 μM), but no significant inhibition of
proliferation was seen on L3.6pl cells at doses up to 100 μM
(Figure 2). The antiproliferative effects on L3.6pl cells were
seen exclusively at very high doses (IC50 = 1000 μM). How-
ever, this effect might be caused by an unspecific cytotoxic
activity of the agent at those concentrations.

Single-Dose Therapy
Twenty-four hours after a single-dose treatment with
ZD6126 (± gemcitabine), extensive tumor necrosis was
seen in six of six and in five of six mice, respectively
(Figure 3, C and D). Only a slim rim of viable tumor cells
was found in the periphery of those tumor nodules. In
contrast, no remarkable tumor necrosis was seen in controls
or under gemcitabine monotherapy (Figure 3, A and B).

Primary Tumor Model (Chronic Treatment)
Longer-term therapy appeared to be well tolerated, and
the assessment of body weight showed no significant dif-
fferences between all treatment groups (Table 1). Regarding
primary tumor growth, a significant reduction of pancre-
atic tumor weight was found in all treatment groups com-
pared to controls (Table 1). As expected out of preliminary
dose–response assays for gemcitabine, tumor growth inhib-
hition in vivo was about 50% compared to controls. How-
ever, tumor weight under combination therapy with ZD6126
and gemcitabine was significantly lower than in all other
treatment groups, reaching approximately 76% growth inhibi-
tion compared to controls (control tumor weight 1320 ±
297 mg; combination therapy 443 ± 61) (Table 1). Concern-
ing metastatic prevention by multiple dosing therapy, mono-
therapy with gemcitabine had no significant effect on liver
and lymph node metastases. But treatment with ZD6126
alone or in combination with gemcitabine led to a sig-
ificant inhibition of liver and lymph node metastases com-
pared to gemcitabine monotherapy and controls (Table 1).
The reduction of lymph node metastasis was not depen-
dent on primary tumor size because gemcitabine mono-
therapy also led to a significant reduction of tumor weight
but did not inhibit lymphatic spread. The frequency of peri-
toneal carcinosis was significantly decreased following
treatment with gemcitabine, ZD6126, and combination
therapy (Table 1).
Liver Metastasis Model (Chronic Treatment)

Regarding the palliative treatment of metastatic disease, ZD6126 and gemcitabine induced a significant reduction of heterotopic tumor mass after the implantation of pancreatic tumor cells into the spleen (weight of spleen). Moreover, the combination of both agents exhibited a significantly stronger growth inhibition than either of the drugs alone (Figure 5A). The size of established liver metastases (Figure 4) was limited by either ZD6126 or gemcitabine monotherapy (Figure 5C), but metastatic tumor burden (weight of the liver) was primarily decreased by gemcitabine or a combination therapy in this model, whereas ZD6126 alone had no significant effect on this parameter (Figure 5B). However, only combination therapy significantly reduced the number of liver metastases when compared to all other treatment groups (Figure 5D). This indicates a therapeutic benefit in combining both agents in our animal model.

Immunohistochemical Analyses

No difference in microvessel density and proliferation index was found in gemcitabine-treated tumors when compared to controls (Figure 6). However, ZD6126-treated tumors (monotherapy and combination therapy) revealed both significantly reduced vascularization (CD31) and cell proliferation (Ki67) compared to gemcitabine treatment and controls. Moreover, the TUNEL assay showed significantly increased apoptotic cell deaths in tumors after gemcitabine, ZD6126, and combination therapy (340 ± 134; 363 ± 127; and 382 ± 52) compared with no treatment (132 ± 75) (Figure 7).

IVM Investigations

During ZD6126 treatment, dorsal skinfold chamber analysis revealed a significant decrease of tumor neovascularization compared with controls (Figure 8A). In vivo microvessel

Table 1. Tumor Growth (Weight) and Metastatic Spread Following 21 Days of Treatment.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Pancreatic Tumors</th>
<th>Liver Metastases</th>
<th>Lymph Node Metastases</th>
<th>Peritoneal Carcinosis</th>
<th>Mean Tumor Weight (mg) ± SD</th>
<th>Mean Body Weight (g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/10</td>
<td>6/10</td>
<td>10/10</td>
<td>7/10</td>
<td>1320 ± 297</td>
<td>19.8 ± 3.7</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>10/10</td>
<td>2/10</td>
<td>10/10</td>
<td>1/10</td>
<td>687 ± 157</td>
<td>19.1 ± 2.2</td>
</tr>
<tr>
<td>ZD6126</td>
<td>8/8</td>
<td>1/8</td>
<td>2/8</td>
<td>0/8</td>
<td>541 ± 201</td>
<td>18.5 ± 1.9</td>
</tr>
<tr>
<td>ZD6126 + gemcitabine</td>
<td>8/8</td>
<td>1/8</td>
<td>3/8</td>
<td>0/8</td>
<td>443 ± 61</td>
<td>17.0 ± 2.0</td>
</tr>
</tbody>
</table>

*P < .02 compared with control.
**P < .001 compared with control.
***P < .05 compared with control.
****P < .01 compared with control.
*****P < .01 compared with gemcitabine.
******P < .005 compared with control.
*******P < .03 compared with ZD6126.
density was significantly reduced by ZD6126 after 1, 4, or 6 days of treatment (Figure 8B).

Discussion
ZD6126 is a potent tubulin-destabilizing agent that induces rapid and reversible morphologic changes in proliferating neoendothelia, whereas normal endothelial cells of non-tumor tissues remain unaffected. In vivo, these conformational changes lead to endothelial cell detachment, tumor vascular congestion, and resultant tumor necrosis, particularly in the central tumor regions that are most reliant on the tumor blood supply [6,10]. Administration of ZD6126 has shown potent antivasular effects at doses well below the MTD, producing little or no classic antitubulin-mediated toxicity (approximately 1/13 of the MTD of other tubulin-binding agents such as combretastatin A-4 phosphate) [6,27]. Moreover, pharmacokinetic studies in mice indicated a short in vivo exposure of ZD6126 phenol due to its fast elimination and, therefore, a rapid reversibility of the ZD6126 effect. Plasma concentration after a single-dose treatment with ZD6126 decreased down to 1/100 of maximum levels within 180 minutes [6].

Central parts of solid tumors are known to show some resistance to conventional radiation therapy and chemotherapy due to tumor hypoxia and elevated interstitial fluid pressure (IFP). Uncontrolled tumor angiogenesis and increasing vascular permeability without an adequate lymphatic drainage lead to elevated IFP and subsequently to lower tissue oxygenation and to reduced drug delivery to the tumor center (e.g., radiosensitisers and chemotherapeutic agents). These mechanisms consequently result in radiation resistance and drug resistance [28,29]. Two different therapeutic approaches are conceivably able to overcome the problem of therapy resistance in central parts of solid tumors. One might be the use of antiangiogenic agents, as reviewed by Jain [29]. Certain antiangiogenic drugs have been shown to normalize the abnormal structure and function of tumor vessels, thereby reducing IFP and inducing more efficient oxygen delivery and drug delivery. The other approach would be the occlusion of tumor vessels by vascular-targeting agents such as ZD6126, inducing a typical central tumor necrosis and leaving only a thin rim of viable tumor cells at the periphery [6,10]. These remaining tumor cells obtain blood supply from surrounding normal vessels and, therefore, should be more sensitive to conventional chemotherapy. Consequently, a combined approach using a vascular-targeting agent and a chemotherapeutic drug may provide a significant therapeutic benefit in solid tumors. In the present study, ZD6126 was used for the first time in combination with gemcitabine, which is the most relevant chemotherapeutic agent for human pancreatic cancer.

In a variety of tumor models (including human xenografts derived from the breast, lung, colon, and stomach), ZD6126 has been shown to induce extensive central

Figure 4. Extended macroscopic liver metastases 29 days after heterotopic tumor cell injection into the spleen (control animal without treatment).

Figure 5. Palliative treatment of metastatic disease (liver metastasis model). (A and B) Tumor burden 29 days after the heterotopic implantation of pancreatic tumor cells into the spleen. (C) Growth inhibition of established liver metastasis. (D) Decreasing number of liver metastases after the combination therapy only. *Treatment versus control, P < .01 (at least); *combination versus ZD6126, P < .05 (at least); §combination versus gemcitabine, P < .05 (at least).
tumor necrosis in both single and multiple dosing regimens [10,11,13,14,30,31]—an action that could be confirmed for human pancreatic cancer in the present study. ZD6126 was found to be associated with a rapid necrosis of established L3.6pl pancreatic tumors 24 hours after single-dose administration. This effect was not seen with gemcitabine therapy alone, although gemcitabine is a potent cytotoxic agent in a variety of solid tumor systems, and L3.6pl cells are highly sensitive to gemcitabine but not to regular doses of ZD6126 phenol, as shown by MTT assays in vitro. This central necrosis in L3.6pl tumors is likely to be the result of rapid tumor vessel disruption as demonstrated by a massive early reduction of tumor vascularization in dorsal skinfold tumors 24 hours after the first ZD6126 administration. Direct effects on tumor cells seem to be unlikely because the in vitro inhibition of L3.6pl cell proliferation was exclusively seen at very high doses of ZD6126 phenol (IC50 = 1000 μM). In contrast, a significant alteration of endothelial cell morphology has been described already at low concentrations (0.07 μM) [19], even much lower than those needed to significantly inhibit endothelial cell proliferation in our study (IC50 ≈ 100 μM).

For multiple dosing regimens, we used a tumor model where pancreatic primary tumors were well established (64–216 mm3) but still organ-confined, to imitate the clinical situation where curative therapy is possible if metastatic disease could be prevented. Therefore, we started treatment early on day 9 in this model. A significant antitumor activity against the primary tumor was evident for both ZD6126 and gemcitabine therapy. Tumor growth delay was obtained most effectively by using a combination of both agents as shown by the lowest primary tumor weight. However, tumor weight obviously underestimates the antitumor effects of ZD6126. Central necrotic areas preserve the measured weight of a primary tumor to some extent without having an impact on the tumor burden. The IVM procedure visualized the multiple dosing effects of ZD6126, which included a rapid and constant reduction in tumor vascularization starting with the first day after the ZD6126 administration. Consistently, histomorphologic and immunohistochemical analyses revealed that ZD6126-mediated antitumor activity was associated not only with central tumor necrosis but also...
with a significant reduction in tumor vessel density and also reduced tumor cell proliferation in viable parts of the tumor. Both ZD6126 and gemcitabine treatments resulted in increased apoptotic cell death in viable parts of those tumors assessed by TUNEL assay. Pharmacokinetic results and our own in vitro studies do not suggest that increased apoptotic cell death and reduced cell proliferation in vivo result from a direct effect of ZD6126 on tumor cells. It is, by far, more likely that there was an indirect effect through tumor hypoxia due to vascular destruction. Consistent with previous observations [22,23], gemcitabine therapy significantly increased tumor cell apoptosis but affected neither the fraction of proliferating tumor cells nor tumor vascularization. Therefore, the synergistic activity of gemcitabine and ZD6126 on tumor growth inhibition in primary tumor models might be explained by the combination of different mechanisms of action such as cytotoxic activity, tumor vessel occlusion, and direct and indirect antiproliferative, proapoptotic, and antiangiogenic effects.

Furthermore, multiple dosing of ZD6126 was associated with a marked antmitastatic activity in primary tumors. Liver and lymph node metastases could be prevented in more than 3/4 of the animals by ZD6126 administration, and this effect was not enhanced by an additional treatment with gemcitabine. Regarding hematogenous metastases such as liver metastases, ZD6126 might act through the destruction of tumor vessels, thereby limiting the route of metastatic spread. Alternatively, a massive reduction of viable tumor volume by ZD6126 per se may be sufficient to decrease the number of metastatic events. Lymph node metastases result from a tumor cell invasion of lymphatic vessels and intralymphatic tumor growths. Lymphatic tumor invasion and lymphangiogenesis occur at the invasive edge of the tumor, where tumor cells proliferate and get in contact with peritumoral lymphatics [32,33]. However, this invasive edge of the tumor should then be identical to the viable rim of proliferating tumor cells in our study. These cells remained unaffected by ZD6126 therapy. Consequently, no effect of ZD6126-dependent tumor necrosis on the frequency of lymph node metastases should be expected. So far, there have been no reports on ZD6126 effects on lymphatic vasculature. However, the significant reduction in lymph node metastases seen in our study could possibly be caused by the additional effects of ZD6126 on tumor-associated lymphatic endothelial cells. A repeated dosing of gemcitabine alone showed no apparent effect on spontaneous lymph node and liver metastases and therefore did not improve the ZD6126 activity of metastatic prevention. Those antimetastatic data for ZD6126 contrast with former studies of antiangiogenic agents assessed in the same tumor model, where single-agent treatment with PTK 787, DC101 (inhibitors of vascular endothelial growth factor signaling), or PKI 166 (an inhibitor of epidermal growth factor receptor signaling) did not affect the development of metastases in L3.6pl pancreatic tumors [22,34]. These findings might indicate an important role of endothelial cell–specific treatment to prevent metastatic spread.

To imitate the clinical situation of the palliative treatment of advanced metastatic disease, we used a model of experimental liver metastases. Here, extended metastatic
were most obvious after the combination of ZD6126 and gemcitabine. This observation highlights the therapeutic potential of combining agents with differing mechanisms of action, particularly when different cell populations within the tumor are targeted (endothelial and tumor cells). Experimental approaches to combine vascular-targeting agents with standard gemcitabine therapy need to be further evaluated.

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References

Conclusion
Our studies confirm previous observations of the single-dose effect of ZD6126, resulting in a central necrosis of established orthotopic pancreatic tumors. Longer-term therapy with repeated doses of ZD6126 in pancreatic tumor models appeared to be well tolerated and resulted in a significantly reduced primary tumor size and in the reduction of lymph node and liver metastases. As shown by in vitro and in vivo experiments, possible mechanisms of ZD6126 involve anti-vascular activity and subsequent antiproliferative and pro-apoptotic effects, whereas a direct activity against tumor cells seems unlikely. Although metastatic prevention by gemcitabine was inefficient in orthotopic tumor settings, the growth inhibition of primary tumors and experimental liver metastases


