Vascular Targeting in Pancreatic Cancer: The Novel Tubulin-Binding Agent ZD6126 Reveals Antitumor Activity in Primary and Metastatic Tumor Models[1]

Axel Kleespies*, Gudrun Köhl[†], Michael Friedrich[†], Anderson J. Ryan[‡], Alan Barge[‡], Karl-Walter Jauch* and Christiane J. Bruns*

*Department of Surgery, Klinikum Grosshadern, Ludwig-Maximilian-University, Munich, Germany; [†]Department of Surgery, University of Regensburg, Regensburg, Germany; [‡]AstraZeneca, Alderley Park, Macclesfield, UK

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 antimetastatic effects of ZD6126 in human pancreatic cancer and reveal benefits of adding ZD6126 to standard gemcitabine therapy.

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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].

ZD6126 is a novel vascular-targeting prodrug developed by AstraZeneca (Macclesfield, UK), which is rapidly converted *in vivo* by serum phosphatases to its active species ZD6126 phenol (*N*-acetylcolchinol), which binds to tubulin and causes microtubular destabilization (Figure 1). Proliferating endothelial cells, such as those of the tumor neovasculature, rely on the tubulin cytoskeleton to maintain their shape. The binding of ZD6126 phenol to tubulin causes rapid morphologic changes in the cell, leading to endothelial cell detachment, tumor blood vessel occlusion, and massive tumor necrosis [6,7]. In contrast, mature endothelial cells of normal blood vessels are less reliant on tubulin due to the presence of a well-defined actin cytoskeleton, a mature basement membrane, and vessel-associated pericytes, which are missing in pathologic tumor vessels. Therefore, morphology of mature endothelial cells is maintained under ZD6126 therapy. The mechanism of ZD6126
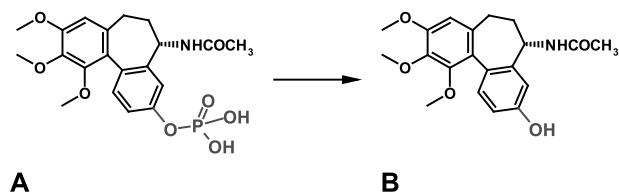
Figure 1. *Chemical structures of (A) the inactive prodrug ZD6126 (N-acetylcolchinol-O-phosphate) and (B) its active metabolite ZD6126 phenol (N-acetylcolchinol).*

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% CO_2 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 (Pharmingen, 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); *Ki*67 (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, 15×10^3 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_{50} 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 mm^3), 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 mm^3 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 for 5 days/week followed by two treatment-free days (75 mg/kg per day, i.p.; $n = 8$); 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 macroscopi-

cally 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 *Ki*67 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. *Ki*67 "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 mm^2).

IVM of Neovascularization

Tumor neovascularization *in vivo* was analyzed using a window model, as described previously [24–26]. Briefly, mice were implanted subcutaneously with 1×10^4 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 [cm^2]) 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 measured, 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 proliferation (MTT assay), IHC quantification of CD31, *Ki*67 and TUNEL, and microvessel density measured by IVM were compared 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 compared 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 (IC$_{50}$ = 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 (IC$_{50}$ = 1000 μM). However, 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 differences between all treatment groups (Table 1). Regarding primary tumor growth, a significant reduction of pancreatic tumor weight was found in all treatment groups compared to controls (Table 1). As expected out of preliminary dose–response assays for gemcitabine, tumor growth inhibition *in vivo* was about 50% compared to controls. However, tumor weight under combination therapy with ZD6126 and gemcitabine was significantly lower than in all other treatment groups, reaching approximately 76% growth inhibition compared to controls (control tumor weight 1320 ± 297 mg; combination therapy 443 ± 61) (Table 1). Concerning metastatic prevention by multiple dosing therapy, monotherapy 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 significant inhibition of liver and lymph node metastases compared to gemcitabine monotherapy and controls (Table 1). The reduction of lymph node metastasis was not dependent on primary tumor size because gemcitabine monotherapy also led to a significant reduction of tumor weight but did not inhibit lymphatic spread. The frequency of peritoneal carcinosis was significantly decreased following treatment with gemcitabine, ZD6126, and combination therapy (Table 1).
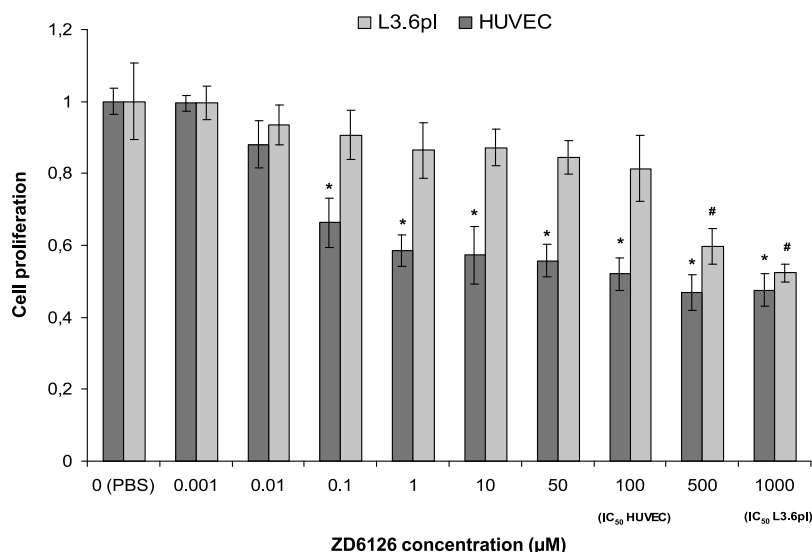

Figure 2. Effect of ZD6126 phenol on the in vitro proliferation of L3.6pl cells and HUVECs using the MTT cell viability test. The IC$_{50}$ concentration was reached at 100 μM for HUVECs and at 1000 μM for L3.6pl cells, respectively. Significant antiproliferative effects: #L3.6pl, P < .01 (at least); *HUVECs, P < .05 (at least).
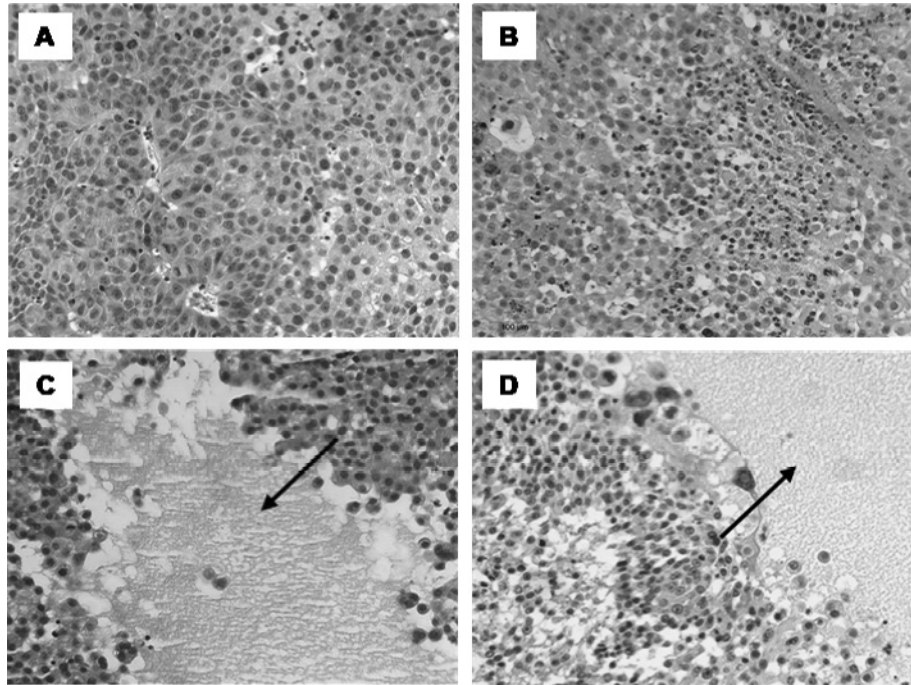
Figure 3. *Development of tumor necrosis 24 hours after single-dose treatment. (A) Control tumor; (B) gemcitabine, 100 mg/kg, i.p.; (C) ZD6126, 150 mg/kg, i.p.; (D) ZD6126, 150 mg/kg, and gemcitabine, 100, mg/kg, i.p.; Arrow = necrotic area.*

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 5*A*). The size of established liver metastases (Figure 4) was limited by either ZD6126 or gemcitabine monotherapy (Figure 5*C*), 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 5*B*). However, only combination therapy significantly reduced the number of liver metastases when compared to all other treatment groups (Figure 5*D*). 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 (*Ki*67) 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 8*A*). *In vivo* microvessel

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

Therapy	Pancreatic Tumors	Liver Metastases	Lymph Node Metastases	Peritoneal Carcinosis	Mean Tumor Weight (mg) ± SD	Mean Body Weight (g) ± SD
Control	10/10	6/10	10/10	7/10	1320 ± 297	19.8 ± 3.7
Gemcitabine	10/10	2/10	10/10	1/10*	687 ± 157[†]	19.1 ± 2.2
ZD6126	8/8	1/8[‡]	2/8[§,¶]	0/8[#]	541 ± 201[†]	18.5 ± 1.9
ZD6126 + gemcitabine	8/8	1/8[‡]	3/8[§,¶]	0/8[#]	443 ± 61[†,¶,**]	17.0 ± 2.0

*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.

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

density was significantly reduced by ZD6126 after 1, 4, or 6 days of treatment (Figure 8*B*).

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 antivascular 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., radiosensitizers 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
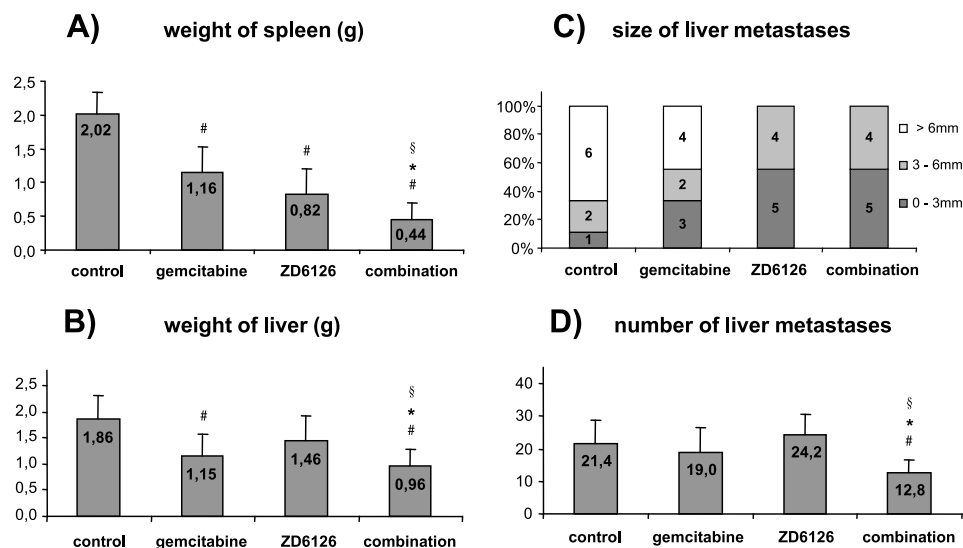

Figure 5. *Palliative treatment of metastatic disease (liver metastasis model). (A and B) Tumor burden 29 days after the heterotopic implantation of pancreatic tumor cells into the spleen. (C) Growth inhibition of established liver metastasis. (D) Decreasing number of liver metastases after the combination therapy only.*
#Treatment versus control, P < .01 (at least); *combination versus ZD6126, P < .05 (at least); §combination versus gemcitabine, P < .05 (at least).
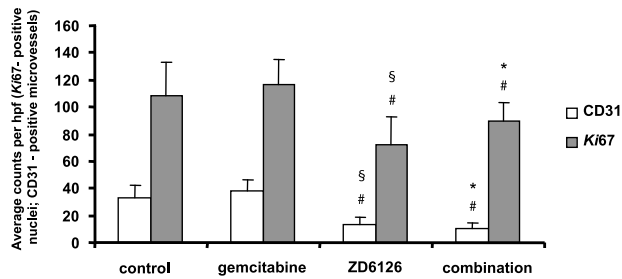
Figure 6. *Immunohistochemical analysis of pancreatic tumors for CD31 and Ki67 expressions (hpf, 0.159 mm²; original magnification, ×100). Data represent the mean values (± SD) of each treatment group (five sections per tumor, five tumors per group, 6–10 hpf per section). #Treatment versus control, P < .05 (at least); *combination versus gemcitabine, P < .05 (at least); §ZD6126 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 re-

duction 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 (IC_{50} = 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 (IC_{50} = 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
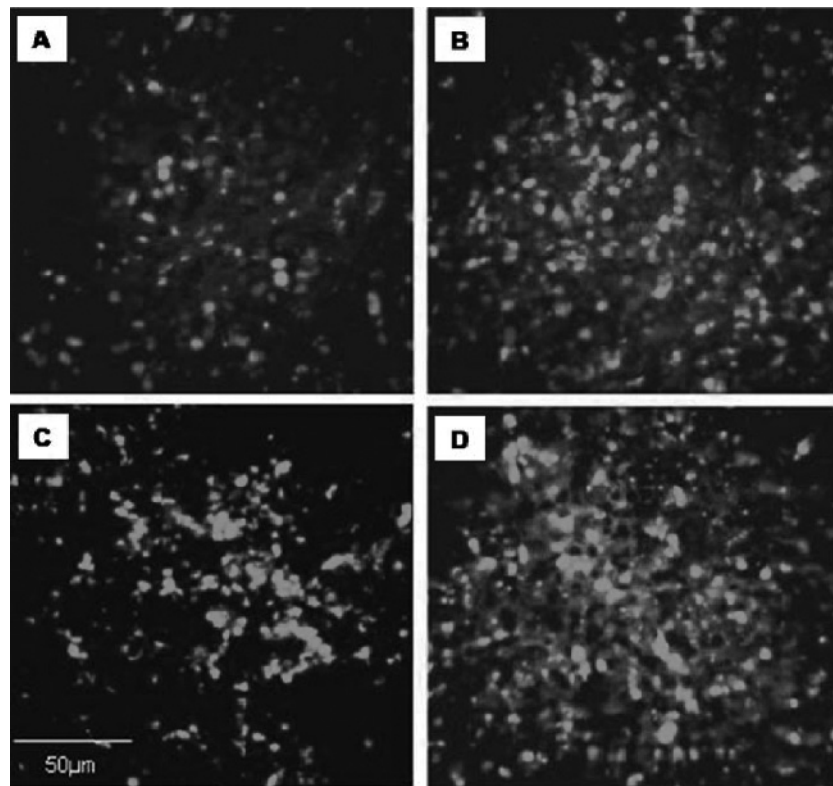

Figure 7. *Apoptotic cell death in primary tumors: mean values (± SD) of TUNEL-FITC–positive cells in (A) control tumors, 132 ± 75; (B) ZD6126 treatment, 363 ± 127*; (C) gemcitabine treatment, 340 ± 134*; (D) ZD6126 + gemcitabine, 382 ± 52*. *Treatment versus control, P < .004 (at least).*
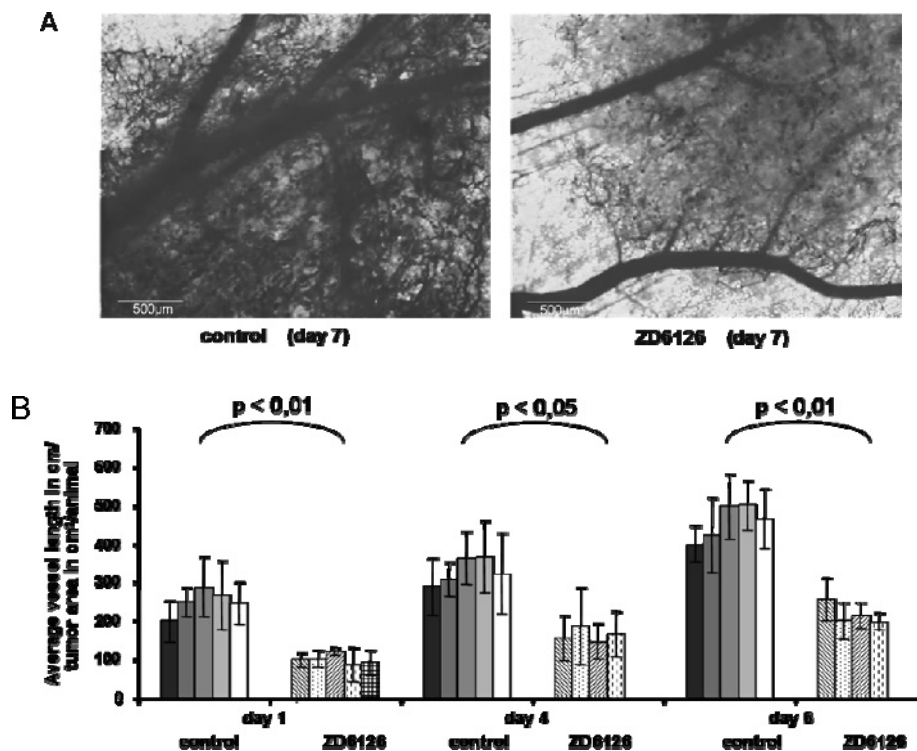
Figure 8. *(A) Visualization of tumor neovasculature in dorsal skinfold chambers by IVM, 7 days after ZD6126 treatment or no treatment. (B) Neovascularization of pancreatic tumors in dorsal skinfold chambers 1, 4, and 6 days after treatment initiation (n = 5) or no treatment (n = 5). Bars present the mean data from 8 to 12 IVM measurements in four random areas per animal (± SD).*

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 antimetastatic 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

disease could be created within 14 days. In contrast, spontaneous liver metastases would occur the earliest on day 23 after orthotopic tumor cell injection. This is about the time when animals become moribund already due to a huge pancreatic tumor mass [17]. In experimental metastatic disease, tumor burden could be significantly controlled by ZD6126 and gemcitabine long-term treatment. In contrast to spontaneous liver and lymph node metastases, gemcitabine revealed significant antimetastatic activities in established metastatic disease, as seen by a reduction of splenic and hepatic tumor mass. The size of established liver metastases was reduced by ZD6126 treatment as well as by gemcitabine treatment, but only the combination of both agents could significantly reduce the number of countable liver metastases. This mechanism becomes plausible because the onset of metastatic growth is a very sudden event in this tumor model, and established liver metastases rapidly gain an efficient blood supply. Both cytotoxic effects (gemcitabine) and antivascular effects (ZD6126) could possibly inhibit the growth of established metastases. However, only the combination of both agents may reduce the number of metastases, probably by eliminating small ones or at least by preserving them in a very early, uncountable, and possibly "dormant" cell cluster status.

The mechanism of ZD6126 action makes it particularly attractive to use this drug in combination with other anticancer approaches, such as certain cytotoxic agents and radiation therapy, as these therapies are most effective against well-vascularized and well-oxygenated areas of the tumor, such as the peripheral rim. Correspondingly, it has been shown that the antitumor effect of ZD6126 could be enhanced when it is used in combination with cisplatin, paclitaxel, and radiation therapy [11,13,14,35]. Gemcitabine is currently licensed for the treatment of locally advanced and metastatic adenocarcinomas of the pancreas. The present study demonstrates for the first time the antitumor and antimetastatic effects of ZD6126 in human pancreatic cancer models. Although metastatic prevention could not be achieved by gemcitabine monotherapy in this study, our results highlight the therapeutic benefits of combining ZD6126 and gemcitabine in advanced metastatic disease, where synergistic effects could be observed compared to an exclusive administration of either drug.

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 antivascular activity and subsequent antiproliferative and proapoptotic 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

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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