Antiangiogenic and antitumor activity of a novel vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor ZD6474 in a metastatic human pancreatic tumor model

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ZD6474 is a novel, orally available inhibitor of vascular endothelial growth factor receptor kinase insert domain receptor/flk-1 tyrosine kinase activity with additional activity against the epidermal growth factor receptor-1 tyrosine kinase. The aim of this study was to evaluate ZD6474, alone and in combination with gemcitabine, in an orthotopic model of metastatic pancreatic cancer. Nude mice (nine to 10/group) were injected orthotopically with 1×10^6 L3.6pl human pancreatic cancer cells. Eight days later, treatment was initiated with vehicle only, gemcitabine (100 mg/kg intraperitoneal twice weekly), ZD6474 (50 mg/kg oral once daily) or a combination of the two treatments. Animals were killed on day 24 posttreatment initiation. The phosphorylation status level of vascular endothelial growth factor receptor-2 and epidermal growth factor receptor as well as the phosphorylation level of AKT and extracellular signal-regulated kinase-1/2 in different human pancreatic carcinoma cells and in human umbilical vein endothelial cells was analyzed by Western blotting. Compared with controls (1231 mg), the mean weight of treated tumors was reduced to 836, 541 and 308 mg in the gemcitabine, ZD6474 and combination groups, respectively. Lymph node metastasis was significantly reduced in both the ZD6474 alone and combined treatment groups, with 3/10 and 1/5 animals developing metastases, compared with 10/10 and 9/9 in the control and gemcitabine groups ($P < 0.003$ and < 0.0003, respectively). Microvessel density and cell proliferation were significantly reduced in the ZD6474 and combined treatment groups ($P < 0.02$). Immunohistochemistry of tumor samples following treatment with ZD6474 resulted in a reduction of the activated and phosphorylated epidermal growth factor receptor, whereas total epidermal growth factor receptor levels were comparable with control tumors. On the basis of Western blot analysis, ZD6474 provides inhibition of tumor angiogenesis through an anti-vascular endothelial growth factor receptor-2 mechanism and inhibition of cancer cell growth through an anti-epidermal growth factor receptor mechanism. ZD6474 decreased primary pancreatic tumor growth and reduced lymph node and liver metastases compared with controls or gemcitabine alone. Tumor growth was inhibited further in animals receiving ZD6474 and gemcitabine in combination.

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Introduction

In the USA, cancer of the pancreas is the fourth leading cause of cancer death, with an estimated 30 700 new cases diagnosed and 30 000 deaths recorded per year. Although progress has been made in the clinical management of these patients, pancreatic cancer remains a major health problem with an estimated 5-year survival rate of only 1–4%. More than 80% of patients have locally advanced or metastatic disease at the time of diagnosis, which excludes the possibility of curative surgery [1–3]. Tumor control in these cases is rarely successful with conventional chemotherapy and median survival is currently less than 6 months [4,5].

The progression of pancreatic cancer is highly dependent on tumor neoangiogenesis. Clinical prognostic data indicate that expression of key proangiogenic factors, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and thymidine phosphorylase, correlate positively with a higher relapse rate and

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shorter patient survival. Furthermore, a high density of microvessels within pancreatic tumors is a prognostic factor for early disease progression [6]. The growth and survival of many pancreatic tumors has also been found to have a dependency upon EGF receptor (EGFR) signaling within tumor cells. Cultured human pancreatic cancer cell lines are known to express high levels of EGFR and produce transforming growth factor (TGF)-α, and human pancreatic cancers overexpress EGFR and all five known ligands [7–10]. Overexpression of EGFR, TGF-α and EGF in human pancreatic tumors correlates with rapidly progressive disease in comparison with tumors that failed to express the receptor and its ligands. The expression of truncated EGFR is associated with inhibition of pancreatic cancer cell growth and enhanced sensitivity to cisplatin [11]. In addition, EGF and TGF-α may contribute to disease progression by inducing VEGF, the major stimulator of angiogenesis in most cancer types [12]. Given the realization that both angiogenesis and EGFR signaling are keys in regulating the tumorgenicity of human pancreatic carcinomas [13–15], strategies to inhibit these processes are being actively explored.

ZD6474 is a novel, orally available inhibitor of VEGF receptor (VEGFR)-2 kinase insert domain receptor/flk-1 tyrosine kinase with additional activity against EGFR tyrosine kinase. With chronic once-daily dosing, ZD6474 has been shown to inhibit tumor growth in a range of models *in vivo* [16]. Given that ZD6474 may inhibit VEGFR and EGFR signaling, we hypothesized in this study that pancreatic cancer progression may be sensitive to treatment and that this activity may be enhanced when combined with a cytotoxic agent. This hypothesis was tested in an orthotopic mouse model of metastatic pancreatic cancer using ZD6474 alone and in combination with gemcitabine, which is used clinically to treat pancreatic cancer.

Materials and methods

Pancreatic cancer cell lines and culture conditions

The high, medium and low metastatic human pancreatic cancer cell lines L3.6pl, AsPC-1 and FG, respectively, were maintained in Dulbecco's minimal essential medium (DMEM), supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, a two-fold vitamin solution (Life Technologies, Grand Island, New York, USA) and penicillin–streptomycin mixture (Flow Laboratories, Rockville, Maryland, USA) to constitute complete DMEM [17]. Confluent human umbilical vein endothelial cells (HUVECs; PromoCell, Heidelberg, Germany) were placed in Endothelial Cell Growth Medium (PromoCell), containing all supplements from the original kit except growth factors. Cultures were free of *Mycoplasma* and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus and lactate dehydrogenase virus (assayed by MA Bioproducts, Walkersville, Maryland, USA). Cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Orthotopic tumor model

Male, 8–12-week-old athymic nude mice (NCr-nu) were purchased from Charles River (Germany). The mice were cared for and housed in laminar flow cabinets under specific pathogen-free conditions in accordance with current regulations and standards of the US Department of Agriculture, the US Department of Health and Human Services, and the US National Institutes of Health.

L3.6pl cells were harvested from subconfluent cultures by treatment with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in Hanks' balanced salt solution. Only single-cell suspensions with greater than 90% viability were used for injections. Orthotopic tumor cell injection was performed as described previously [17]. Mice were injected orthotopically with 1×10^6 L3.6pl human pancreatic cancer cells (day 0). After 8 days, animals received ZD6474 (50 mg/kg/day oral; $n = 10$), gemcitabine (100 mg/kg twice weekly intraperitoneal; $n = 9$), ZD6474 plus gemcitabine ($n = 5$; gemcitabine administered 1 h after ZD6474) or vehicle (1% polysorbate 80) alone ($n = 10$). Animals were killed 24 days after starting treatment. The size and weight of primary tumors were determined, and a dissecting microscope was used to assess macroscopically visible liver lesions, which were counted and processed for immunohistochemistry to confirm the metastatic nature of the lesion by histology. Therefore, five vertical sections of each liver were stained for hematoxylin and eosin to determine whether the counted visible liver lesions were indeed liver metastases. All macroscopically enlarged regional (celiac and paraaortal) lymph nodes were harvested and processed for hematoxylin and eosin staining to confirm that the lymph node enlargement was indeed the result of metastatic spread.

Immunohistochemical analysis

For histology and immunohistochemistry, one part of the tumor tissue was formalin-fixed and paraffin-embedded; another part was embedded in OCT compound (Miles, Elkhart, Indiana, USA), snap-frozen in liquid nitrogen and stored at −70°C. Primary tumors were evaluated for CD31 expression (an indicator of blood vessel density) using frozen 8–10-μm sections prepared and mounted on positively charged slides (Fisher Scientific, Schwerte, Germany). Sections were air-dried and fixed in cold acetone for 5 min, acetone/chloroform for 5 min, acetone

again for 5 min, and then washed in phosphate-buffered saline. CD31 staining was performed as described previously [17], using 3-amino-9-ethylcarbazole as a chromogen for visualization, and counterstained with hematoxylin. Paraffin-embedded tissue sections of primary tumors were used to determine Ki67 (a proliferation marker), M30 cytoDEATH (a marker of apoptosis), VEGF expression [17] and basic fibroblast growth factor [17] expression. Tissues were embedded in paraffin and 4–6-μm sections were prepared and mounted on positively charged Superfrost slides (Fisher Scientific). Sections were dried overnight, deparaffinized in xylene, and treated in 100, 95 and 80% ethanol (v/v, in distilled water), before rehydrating in phosphate-buffered saline. Ki67 'antigen retrieval' was achieved by microwaving tissue sections for 5 min, and immunohistochemical analysis was performed using a streptavidin–biotin method with a 3,3′-diaminobenzidine chromogen and counterstaining with hematoxylin.

Quantification of microvessel density was performed on 10 random high-power fields (0.159 mm^2 at $\times 100$ magnification) using a Sony 3-chip camera (Sony Corporation of America, Montvale, New Jersey, USA) mounted on a Zeiss universal microscope (Carl Zeiss, Thornwood, New York, USA) and Optimas Image Analysis software (Bioscan, Edmond, Washington, USA). Microvessels were quantified according to a method described previously [17]. The mean number of Ki67-positive cells was calculated by dividing the entire amount of Ki67-positive cells by the number of high-power fields that were counted. The amount of apoptotic areas per tumor (M30 cytoDeath positive areas) was analyzed at $\times 20$ magnification using Optimas Image Analysis software and was expressed as the ratio of apoptotic areas in μm^2 to 1 mm^2 total tumor area.

For all immunohistochemical studies, five sections from five to six different primary tumors per group were assessed and six to 10 high-power fields per section were included in the analyses. All immunohistochemical analyses were performed blinded.

In addition, some paraffin-embedded tissues from control and ZD6474-treated tumors were used for identification of total EGFR (monoclonal mouse anti-human IgG1 EGFR clone 30; Biogenex, San Ramon, California, USA), some frozen sections were used for immunohistochemical staining of activated EGFR (mouse anti-human EGF-R IgG1 (activated form), which reacts specifically with the activated and phosphorylated human EGFR (Chemicon, Temecula, California, USA).

Dorsal skin-fold chamber

Tumor angiogenesis was analyzed *in vivo* using the transparent dorsal skin-fold chamber model, as described previously [18,19]. Chambers were inoculated with 1×10^5 L3.6pl cells. The day after tumor inoculation, mice were treated orally with vehicle (control) or ZD6474 (50 mg/kg/day). Intravital microscopy (Zeiss Axiotech Vario microscope, Göttingen, Germany) was performed daily. For the analysis of angiogenesis, the entire tumor was examined by intravital microscopy and four to 12 images per tumor were recorded by video (modified Sony 3CCD Color Video Camera, AVT Horn, Aalen, Germany). Microvascular density (combined vessel length/tumor area in cm^{-1}) was then measured using Image J software (Wayne Rasband, Version 1.25s; National Institutes of Health, Bethesda, Maryland, USA) by generating horizontal grid lines 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 μm/pixel was used to calculate the actual vessel diameter.

Western blotting

Confluent human pancreatic carcinoma cell lines L3.6pl, FG and AsPC-1 were cultured in serum-reduced DMEM (Gibco, Invitrogen, Karlsruhe, Germany) for 24 h. Confluent HUVECs (PromoCell) were placed in Endothelial Cell Growth Medium (PromoCell) containing all supplements from the original kit except for growth factors for 24 h. Following this conditioning period, the cells were stimulated with 20 ng/ml human recombinant VEGF-A_{165} (R&D Systems, Wiesbaden, Germany) for 10 min or with 50 ng/ml human EGF (Sigma, Taufkirchen, Germany) for 10 min, resuspended in ice-cold radioimmunoprecipitation buffer supplemented with the cocktail of protease/phosphotase inhibitors (Roche, Mannheim, Germany) to a final concentration of about 10^7–10^8 cells/ml. Cells were incubated on ice for 10 min and centrifuged at 14 000*g* at 4°C for 10 min. An equal amount of protein was run on polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore, Boston, Massachusetts, USA) and detected using an enhanced chemiluminescense system (Amersham, Braunschweig, Germany). Phosphorylated VEGF-R2 and total VEGF-R2, phosphorylated EGFR and total EGFR, phosphorylated AKT and total AKT, phosphorylated extracellular signal-regulated kinase mitogen-activated protein kinase, and total mitogen-activated protein kinase antibodies were purchased from Cell Signaling (New England Biolabs, Frankfurt am Main, Germany). Anti-β-actin antibody was purchased from Sigma. All antibodies were used according to the manufacturer's instructions.

Statistical analysis

Pancreatic tumor weight, body weight, and quantification of Ki67, CD31 and apoptotic areas were compared using one-way analysis of variance with a Student–Newman–Keuls multiple comparisons test (InStat 3.0 Statistical Software; Graphpad Software, San Diego, California, USA). The relative rates of liver and lymph node

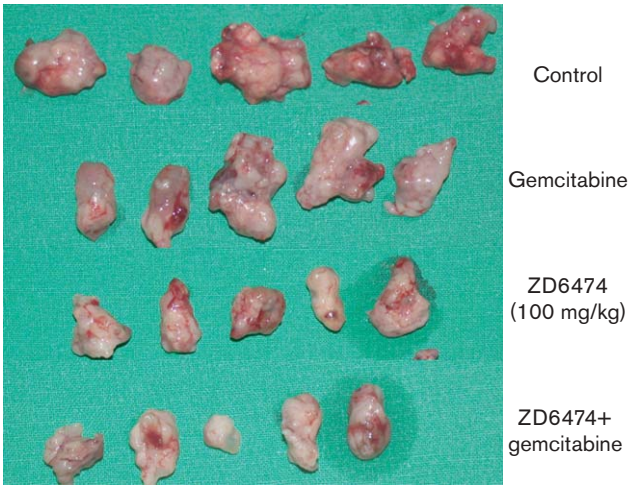
metastases within groups were compared by Fisher's exact test. All analyses were performed with $P < 0.05$ considered to be significant.

Results

Inhibition of tumor growth and metastasis

Compared with controls, therapeutic treatment resulted in reduced tumor growth, most notable in those treated with a combination of ZD6474 and gemcitabine (Fig. 1). Secondary tumors were frequently seen in control animals with all vehicle-treated animals exhibiting lymph node metastases (Table 1). Although gemcitabine alone affected the primary tumor growth it had no effect on the incidence of lymph node metastases. In comparison, the incidence of liver and lymph node metastases was significantly reduced in the group treated with ZD6474 alone. Similarly, significant inhibition of metastases to liver or lymph node metastases was seen in animals receiving the ZD6474/gemcitabine combination. ZD6474 therapy was well tolerated, as indicated by assessment of body weight over the course of the study; the average body weight did not differ significantly from the control animals in any treatment group. The animal experiments were repeated twice. The first experiment was considered as a pilot experiment with five animals per group. The in-vivo results of the pilot study were comparable with the in-vivo results demonstrated in Table 1.

Fig. 1



Primary pancreatic tumor size following therapy with ZD6474, gemcitabine or a combination of the two agents.

Effects on vascular development, cell proliferation and cell death

In the proliferating areas at the periphery of the tumor, microvessel density (measured by CD31 staining) was significantly reduced by ZD6474 treatment (Figs 2a and 3). Combined ZD6474/gemcitabine therapy resulted in slightly greater reductions in microvessel density accompanied by extensive apoptosis-related cell death, as shown by staining for M30 cytoDEATH (Figs 2a and 4).

The results of Ki67 staining showed that the relative number of proliferating tumor cells was substantially lower in mice treated with ZD6474 or ZD6474 plus gemcitabine, when compared with control tumors (Figs 2a and 3). Results from in-vitro pancreatic tumor cell proliferation assays, however, did not reflect these findings, even at high concentrations (1–10 µmol/l ZD6474) only a moderate antiproliferative effect on L3.6pl cells was seen (data not shown). Gemcitabine had no statistically significant effect on tumor cell proliferation either *in vivo* or *in vitro*. Immunohistochemical analysis for the activated, tyrosine-phosphorylated EGFR in L3.6pl tumors demonstrated specific immunoreactivity in control tumors as compared with a reduced immunoreactivity following ZD6474 treatment, whereas total EGFR levels were comparable between control and treated tumors (Fig. 2b).

Skin-fold chamber analysis

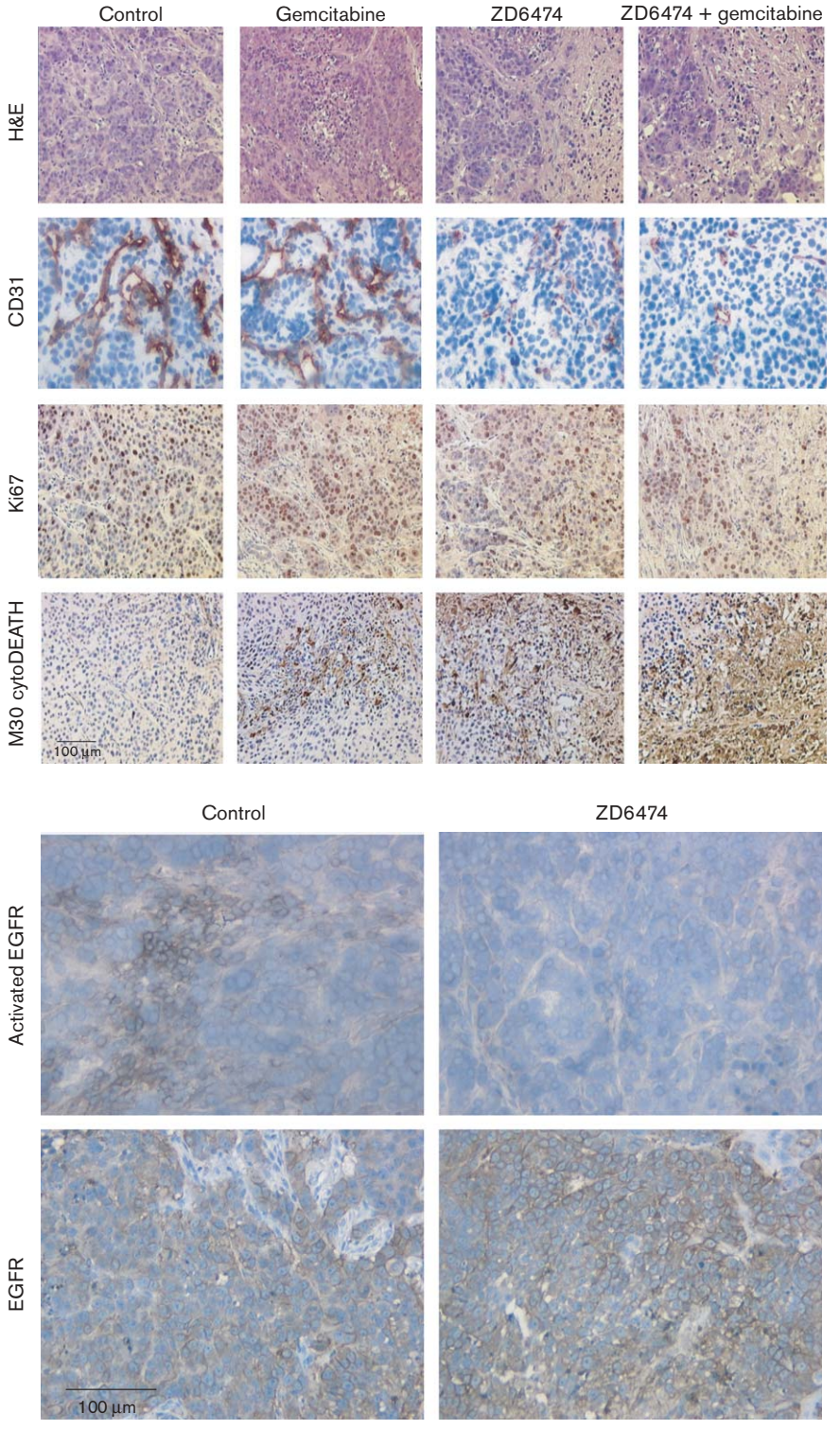
Visualization of tumor vasculature in mice treated with ZD6474 showed significantly fewer blood vessels than tumors growing in the dorsal skin-fold chamber of control

Table 1 **In-vivo primary pancreatic tumor growth and metastases**

Therapy	Incidence				Tumor weight average ± SD (mg)	Body weight average ± SD (g)
	Pancreatic tumor	Liver metastases	Lymph node metastases	Peritoneal carcinosis		
Control	10/10	6/10	10/10	3/10	1231 ± 290	21.4 ± 2.2
Gemcitabine (100 mg/kg twice weekly intraperitoneal)	9/9	4/9	9/9	3/9	836 ± 291[a]	21.7 ± 2.1
ZD6474 (50 mg/kg/day oral)	10/10	1/10	3/10[b]	1/10	541 ± 188[a,c]	19.2 ± 2.9
ZD6474 plus gemcitabine	5/5	0/5[d]	1/5[e]	0/5	308 ± 129[a,c,f]	19.3 ± 3.4

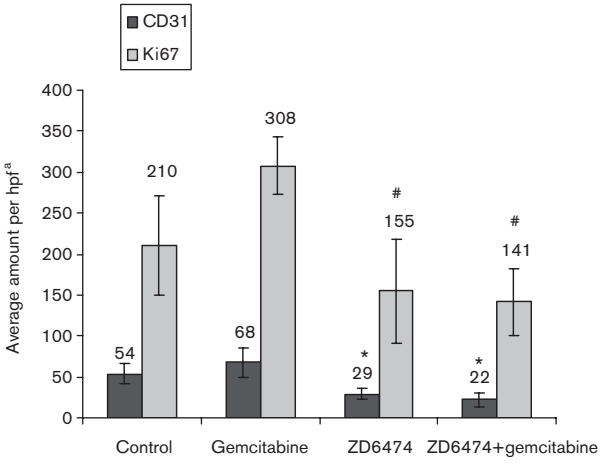
[a]Gemcitabine versus control, $P<0.01$; ZD6474 versus control, $P<0.0001$; combination versus control, $P<0.00001$, unpaired Student's *t*-test.
[b]ZD6474 versus control, $P<0.003$.
[c]Gemcitabine versus ZD6474, $P<0.02$; combination versus gemcitabine, $P<0.001$, unpaired Student's *t*-test.
[d]Combination versus control, $P<0.04$, Fisher's exact test.
[e]Combination versus control, $P<0.0003$, Fisher's exact test.
[f]ZD6474 versus combination, $P<0.02$, unpaired Student's *t*-test.

Fig. 2



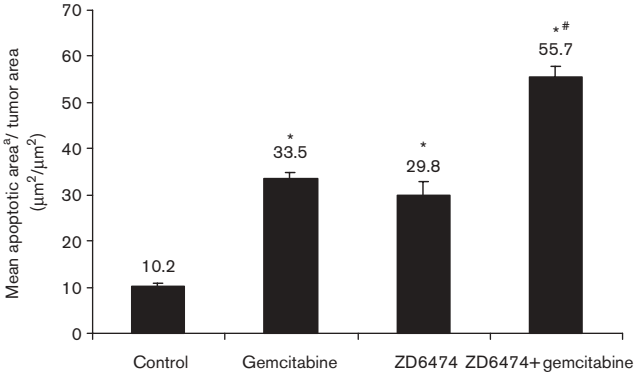
(a) Hematoxylin and eosin (H&E) staining and immunohistochemical analysis of primary pancreatic tumors for Ki67 (proliferation), CD31 (microvessel density) and M30 cytoDEATH (apoptosis). (b) Immunohistochemistry of activated epidermal growth factor receptor (EGFR) and total EGFR in primary pancreatic tumors.

Fig. 3



Immunohistochemical analysis of primary pancreatic tumors for CD31 (microvessel density) and Ki67 (proliferation). *CD31, control versus ZD6474 or ZD6474 and gemcitabine, $P<0.02$. #Ki67, control versus ZD6474 or ZD6474 and gemcitabine, $P<0.02$. [a]High power field at $\times 100$ magnification $= 0.159\,mm^2$.

Fig. 4



Immunohistochemical analysis of primary pancreatic tumors for M30 cytoDeath (apoptosis). *Control versus ZD6474 or gemcitabine, $P<0.02$; control versus ZD6474 and gemcitabine, $P<0.001$. #ZD6474 and gemcitabine versus ZD6474 or gemcitabine, $P<0.02$. [a]M30 cytoDEATH positive area.

animals (Fig. 5a and b). Image analysis data showed that the combined vessel diameter was significantly reduced in tumors of the ZD6474-treated animals compared with controls. Each data point represents the average combined vessel length in cm/tumor area in cm^2 per animal consisting of three to 12 singular measurements. After 6 days of treatment, the average combined vessel length in cm/tumor area in cm^2 per animal was significantly less than in control animals.

Expression of growth factor receptors and their downstream signaling in human pancreatic cancer cells

To investigate the molecular mechanisms responsible for the biological effect of ZD6474 on pancreatic carcinoma cells, we analyzed the expression and phosphorylation of drug-modulated cell targets such as VEGF-R2 and EGFR, as well as the main steps of their signal transduction pathways ERK1/ERK2 and AKT in L3.6pl, AsPC-1 and FG cells. The expression and activity of VEGF-R2 and EGFR was determined in the presence or absence of the cognate ligands VEGF and EGF. As seen in Fig. 6a and b, in the absence of stimulation, autophosphorylation of the VEGF-R2 and EGFR was nearly undetectable in all cell lines. Interestingly, in the presence of 20 ng/ml VEGF, VEGF-R2 phosphorylation was also undetectable (Fig. 6a). In contrast, 50 ng/ml EGF was capable of inducing detectable EGFR phosphorylation in L3.6pl, but not in FG and AsPC-1 cell lines (Fig. 6b). Total VEGF-R2 or EGFR was found in all cell lines, the expression level of these receptors was most pronounced in L3.6pl cells. Together, these results identify EGFR as a main target for the action of ZD6474 on L3.6pl tumor cells.

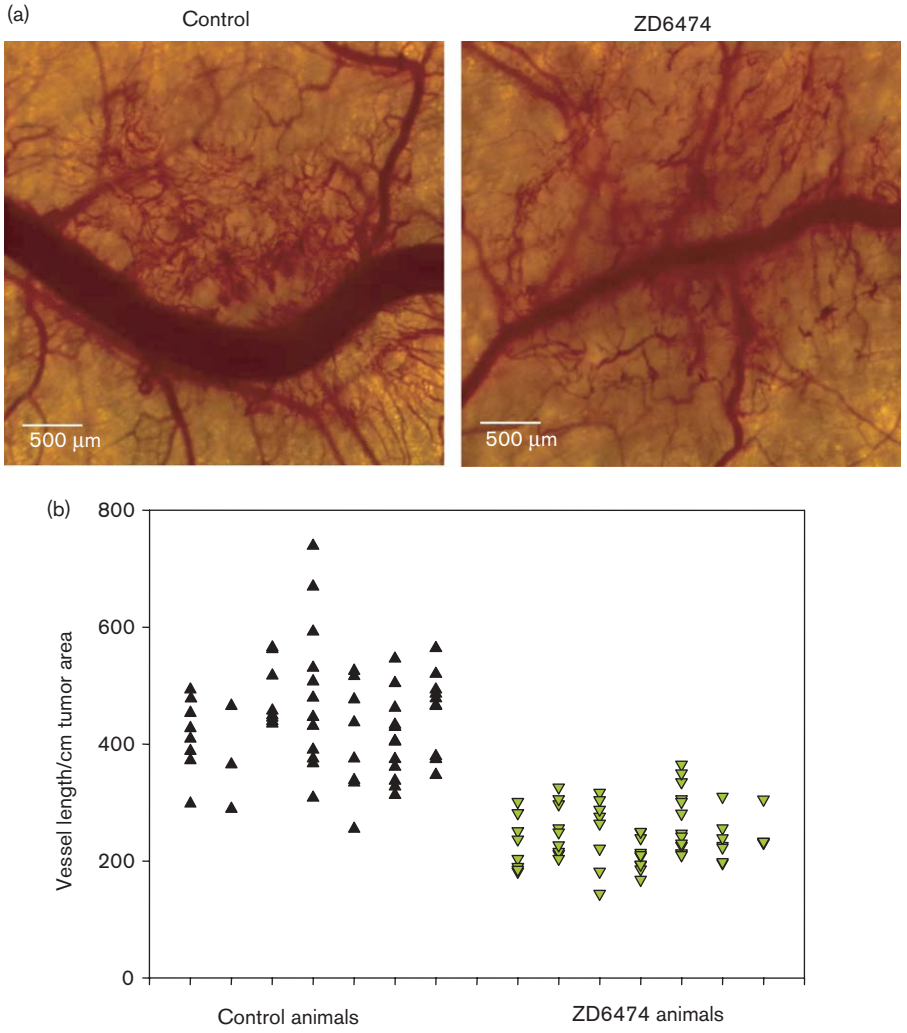
Furthermore, to completely differentiate the two different target points of ZD6474, we investigated the AKT and extracellular signal-regulated kinase (ERK)-1/2 pathways in these cell lines. Interestingly, detectable EGF- and VEGF-mediated activation of the AKT pathway occurred in FG and AsPC-1, but not in L3.6pl cell line (Fig. 6a and b), whereas activation of the ERK1/ERK2 pathway – under the same stimulating conditions – occurs independently of the cell line used with the most marked effect of EGF on L3.6pl cells (Fig. 6b). The VEGF-induced AKT and ERK1/ERK2 signaling cascade is mediated via activation of VEGF-R2; however, none of the used pancreatic cancer cell lines in our study expressed active VEGF-R2 (Fig. 6a).

Therefore, we assume that ZD6474 treatment might cause an inhibition of tumor cell growth and induction of apoptosis in those human cancer cells that express active EGFR, e.g. L3.6pl, suggesting that the potential antiproliferative effect of ZD6474 on tumor cells is probably attributable to the inhibition of the EFGR–ERK1/ERK2 signaling pathway.

Expression of growth factor receptors and their downstream signaling in human umbilical vein endothelial cells

We next investigated which growth factor receptors and their downstream signal pathways are expressed in endothelial cells and which of them would be then the target of ZD6474. The expression and activity of VEGF-R2 and EGFR was determined in the presence or absence of VEGF and EGF. The level of VEGF-R2 was significantly higher compared with the EGFR protein level under the same loading conditions (Fig. 7a and b). Treatment of HUVECs with 20 ng/ml VEGF resulted in a

Fig. 5



Microvessel density following 6 days therapy with ZD6474 in L3.6pl pancreatic tumors growing in dorsal skin-fold chambers. (a) Intravital microscopy image. (b) Assessment of combined vessel length; each data point represents the average combined vessel length in cm/tumor area in cm^2 per animal consisting of three to 12 singular measurements.

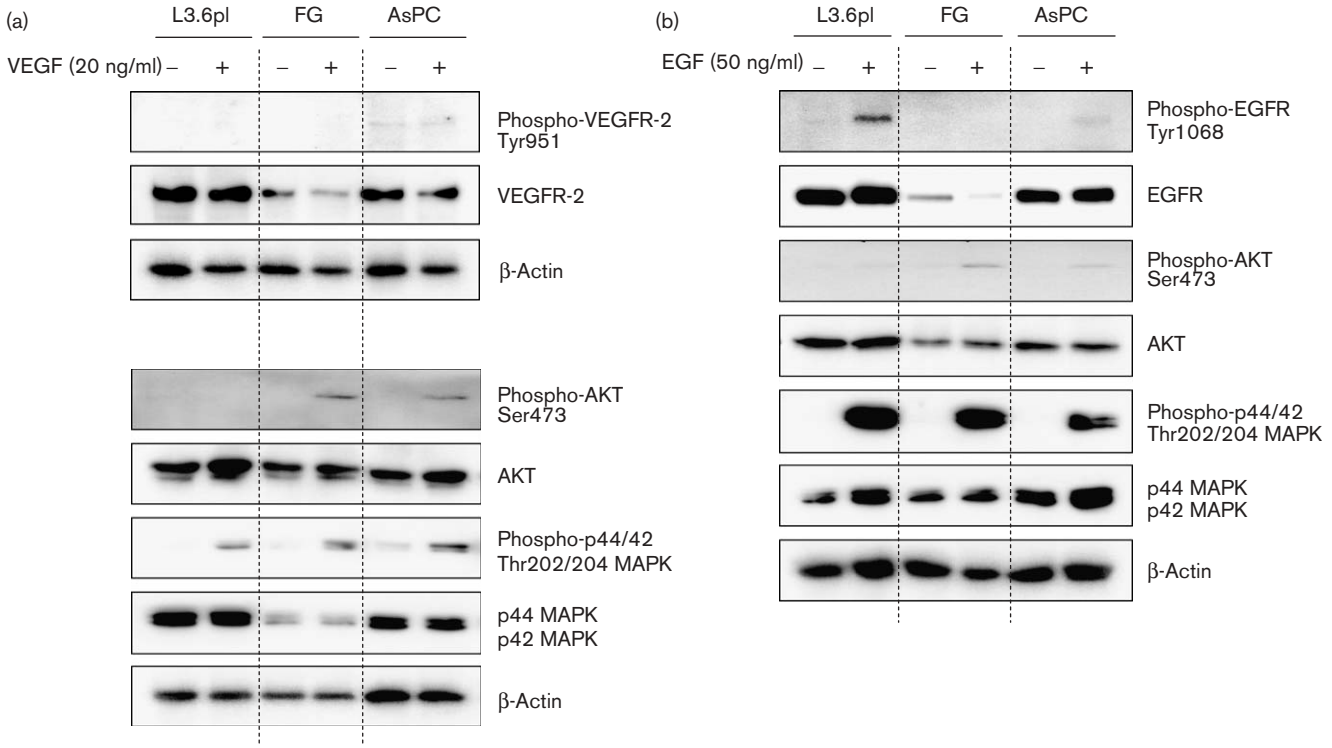
significant increase in VEGF-R2-Tyr951 phosphorylation. Upon stimulation with 50 ng/ml EGF, we also found phosphorylation of EGFR at Tyr1068 in HUVECs, but not in unstimulated cells (Fig. 7a). Interestingly, the growth factor-mediated activation of AKT and ERK1/ERK2 in HUVECs only occurred under VEGF-stimulated conditions (Fig. 7c).

On the basis of these findings, we can expect that in addition to its anti-EGFR efficacy in tumor cells, ZD6474 can also potentially inhibit VEGF-R2–AKT and VEGF-R2–ERK1/ERK2 signaling in endothelial cells, which may offer added therapeutic benefit in tumors with VEGF-R2-dependent endothelial cell proliferation and survival.

Discussion

Cancer of the pancreas continues to pose a major health problem with incidence rates currently matching mortality rates [1,2]. This is largely owing to difficulties in diagnosis, the aggressiveness of pancreatic cancers, early metastasis and the lack of effective therapies [1,2]. Gemcitabine, a deoxycytidine analog, is the most widely used first-line therapy for patients with advanced pancreatic cancer. Treatment with this agent can achieve significant improvements in response rate and survival over 5-fluorouracil therapy, but the median survival for metastatic disease remains less than 6 months. Cancer of the pancreas continues to pose a major health problem with incidence rates currently matching mortality rates [1,2].

Fig. 6



Analysis of protein expression in L3.6pl, FG and AsPC-1 human pancreatic carcinoma cells. Serum-starved cells were treated or not for 10 min with 20 ng/ml human recombinant vascular endothelial growth factor (VEGF) (a) or with 50 ng/ml human epidermal growth factor (EGF) (b). The cells were collected and subjected to lysis, and phosphorylated (phospho) and total proteins were detected by Western blot analysis. β-Actin is shown as a loading control. MAPK, mitogen-activated protein kinase.

In this study, we have demonstrated the potential efficacy of a novel tyrosine kinase inhibitor, ZD6474, alone and in combination with gemcitabine, in a model of pancreatic carcinoma. ZD6474 inhibits VEGFR tyrosine kinase and has additional activity when compared with EGFR tyrosine kinase [16].

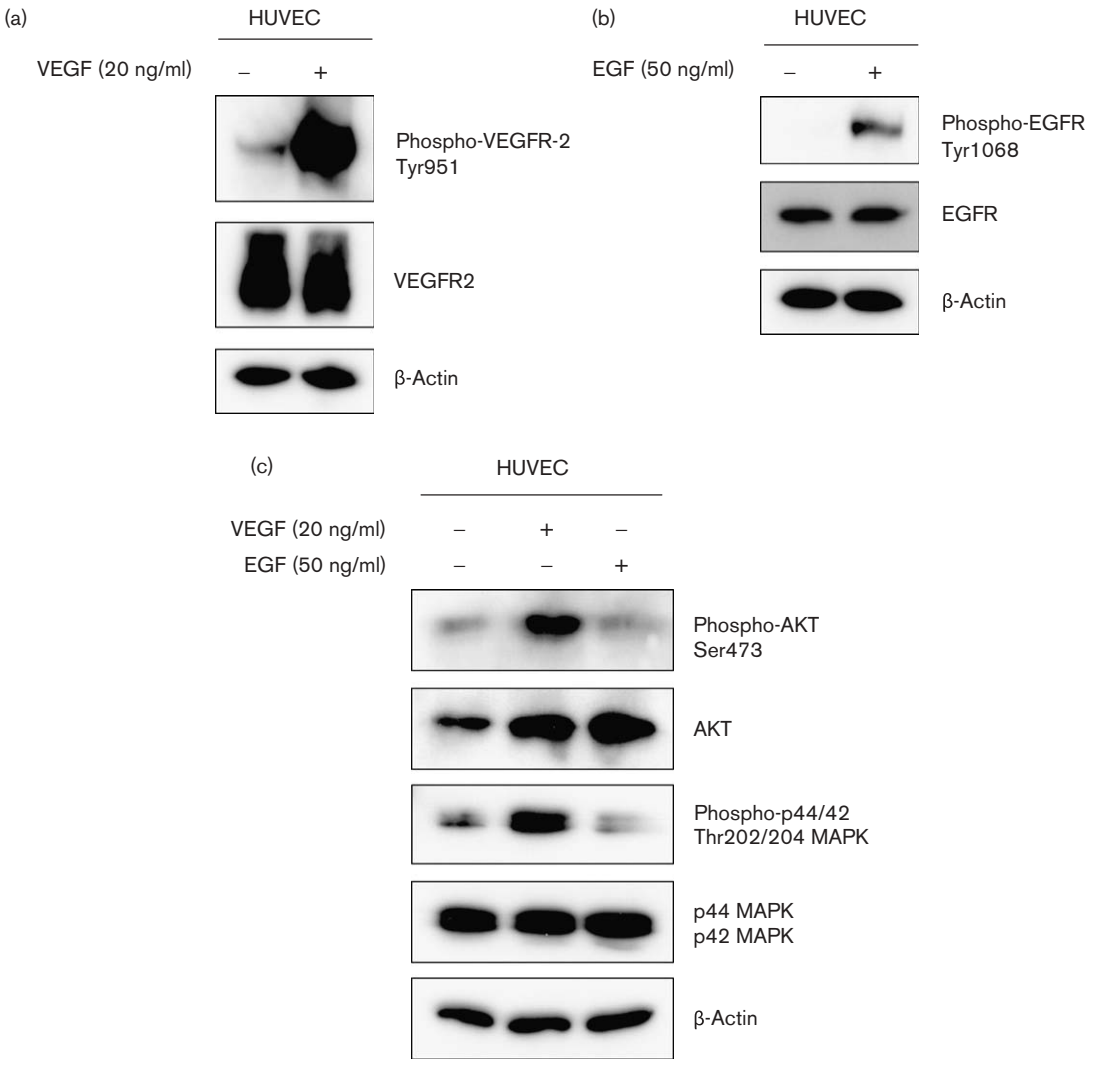
VEGF and VEGFR-2 are critical regulators of endothelial cell proliferation, migration, differentiation and survival, and therefore play a central role in tumor angiogenesis [20–25]. A variety of different inhibitors of VEGF activity have been developed, including humanized neutralizing anti-VEGF monoclonal antibodies [26], soluble VEGFRs [27], antisense VEGF mRNA-expressing constructs [28], VEGF–toxin conjugates [29] and inhibitors of VEGFR function [16,30].

VEGF has been implicated in the angiogenesis of human pancreatic cancer [31], and VEGF expression has been shown to be associated with a poor prognosis in this tumor type [32–34].

Previous studies with ZD6474 have demonstrated inhibition of physiological and pathological angiogenesis *in vivo*, following chronic once-daily oral administration [16]. ZD6474 (12.5–100 mg/kg/day) induces dose-dependent inhibition of tumor growth in a range of histologically distinct human xenograft models (breast, lung, prostate, colon, ovary and vulval), in addition to activity in syngeneic (B16-F10-AP3 melanoma, Lewis lung carcinoma), autochthonous (7,12-dimethylbenzanthracene-induced mammary carcinogenesis), orthotopic (renal murine cell carcinoma) and metastatic (PC14PE6 lung adenocarcinoma, MDA-MB-435-S1/HGF melanoma) tumor models [16,35–39]. This broad spectrum of antitumor activity is consistent with an effect on tumor vascular development or neovascular survival, which is a common feature in the progression of each model. Although ZD6474 may also inhibit EGFR signaling, it has been proven that its activity against kinase insert domain receptor alone is sufficient to confer significant antitumor activity in preclinical tumor models that have intrinsic or acquired resistance to EGFR antagonists [40,41].

In the L3.6pl orthotopic tumor model, treatment with ZD6474 alone resulted in significant inhibition of primary tumor growth. Immunohistochemistry of orthotopically

Fig. 7



(a–c) Analysis of protein expression in human umbilical vein endothelial cells (HUVECs). Growth factor-starved cells were treated or not for 10 min with (a) 20 ng/ml human recombinant vascular endothelial growth factor (VEGF) or with (b) 50 ng/ml human epidermal growth factor (EGF). The cells were collected and subjected to lysis, and phosphorylated (phospho) and total proteins were detected by Western blot analysis. β-Actin is shown as a loading control. EGFR, EGF receptor; MAPK, mitogen-activated protein kinase.

implanted tumors and the use of skin-fold chambers to monitor blood vessel development closely show that the principal mechanism of action appears to be inhibition of vascularization, thereby inhibiting the growth and development of primary tumors. Microvessel density was significantly reduced in the proliferating area of pancreatic tumors treated with ZD6474 or combination therapy as compared with tumors treated with gemcitabine or control tumors. We also observed *in vivo* a significant reduction of tumor cell proliferation and an increase in apoptotic areas. As ZD6474 did not have a potent antiproliferative/apoptotic effect on L3.6pl tumor cells *in vitro* (data not shown), we assume that the effects on tumor cells *in vivo*, following treatment with ZD6474, are a consequence of inhibiting tumor vascularization. This is most likely attributable to direct inhibition of VEGFR signaling, although we cannot preclude the possibility that inhibition of EGFR *in vivo* by ZD6474 could have also contributed to the therapeutic effect observed: EGFR-selective kinase inhibitors have also been found to downregulate VEGF expression significantly and reduce microvessel density in this particular tumor model [33].

To differentiate the two different target points of the drug in pancreatic cancer we analyzed in the presence or

absence of VEGF and EGF the expression and activity of VEGF-R2 and EGFR in different human pancreatic carcinoma cell lines and in HUVECs. Interestingly, we could not identify any active VEGF-R2 or phosphorylated AKT upon stimulation with VEGF in L3.6pl cells. Furthermore, in FG and AsPC cells the amount of active VEGF-R2 and phosphorylated AKT was fairly low. Therefore, our results suggest that the antitumor activity of ZD6474 found *in vivo* is rather partly attributable to the inhibition of the EGFR–ERK1/ERK2 signaling pathway in L3.6pl cancer cells leading to an inhibition of tumor cell proliferation and production of tumor cell-derived VEGF. The withdrawal of the survival factor VEGF causes inhibition of proliferation and apoptosis of endothelial cells. So, the ZD6474-mediated inhibition of the EGFR–ERK1/ERK2 signaling pathway already contains two modes of action: a direct antiproliferative effect on tumor cells and an indirect antiangiogenic effect. In addition to its anti-EGFR efficacy, ZD6474 can also inhibit VEGF-R-2–AKT and VEGFR-2–ERK1/ERK2 signaling in endothelial cells, which offers added therapeutic benefit as a direct antiangiogenic strategy in tumors with VEGFR-2-dependent endothelial cell proliferation and survival.

ZD6474 also significantly inhibited development of metastases in the L3.6pl pancreatic cancer model. We have examined previously the effect of DC101, an antibody that binds to VEGFR-2 to prevent ligand activation, on the primary tumor growth and metastasis of L3.6pl cells growing orthotopically in nude mice [42]. Treatment with DC101 alone inhibited primary tumor growth significantly (by around 60%, comparable with the effect of ZD6474), but had little effect on lymph node metastasis. PTK787 (75 mg/kg), a VEGFR tyrosine kinase inhibitor, has also been examined in this model and again found to inhibit tumor growth similarly, but did not inhibit lymph node metastases, even when combined with gemcitabine [34]. Blockade of the EGFR signaling pathway by daily oral administration of the EGFR tyrosine kinase inhibitor, PKI166 (100 mg/kg) also inhibited growth of L3.6pl pancreatic tumors to a similar extent to ZD6474, but again did not inhibit lymph node metastases significantly [33]. Interestingly, when a VEGFR and EGFR tyrosine kinase inhibitor were combined [PKI166 (50 mg/kg) with PTK787 (50 mg/kg)], the combination did not have superior or additive therapeutic effects on tumor volume or any effect on lymph node metastases [43]. Collectively, these data suggest that the effect of ZD6474 on lymph node metastases is a novel observation, which may not simply be attributed to an inhibition of primary tumor growth, via prevention of VEGFR-2 signaling, or from having additional activity versus EGFR tyrosine kinase. These results are in agreement and extend to those of Giannelli *et al.* [35], demonstrating that ZD6474 also inhibits adhesion, migration and invasion of human hepatocellular carcinoma cells *in vitro*. Furthermore, daily oral dosing with ZD6474 has also been found to inhibit distant metastasis from established lung tumors and to limit the growth of non-small-cell lung cancer metastases [36]. The molecular mechanisms that contribute to the antimetastatic effects of ZD6474 via lymphatics, however, remain unclear.

One potential explanation might be that ZD6474 inhibits additional VEGFR family members, when compared with DC101 (VEGFR-2 specific) and PTK787. Interestingly, it has been shown that PTK787 does not appear to inhibit Flt-4 (VEGFR3) in comparison with VEGFR-2, whereas recombinant enzyme data generated with ZD6474 show that the difference between inhibition of Flt-4 and VEGFR-2 is less than three-fold [16,44]. Flt-4, the VEGF-C and VEGF-D receptor, is important for lymphangiogenesis. A number of clinical links exist between VEGF-C and VEGF-D expression and metastasis and prognosis, and experimentally it has been shown that lymph node metastasis can be reduced with an Flt-4 antibody [45].

In conclusion, once-daily, oral treatment with ZD6474 in this model of metastatic pancreatic carcinoma resulted in significant reduction of primary tumor growth and metastatic spread. When combined with gemcitabine, greater inhibition of tumor growth was observed with highly significant effects on the development of secondary tumors, making this combination of potential interest to the treatment of pancreatic cancer. Phase II clinical investigation of ZD6474 as monotherapy or in combination with certain cytotoxic agents is ongoing.

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References

1 Brand RE, Tempero MA. Pancreatic cancer. *Curr Opin Oncol* 1998; **10**:362–366.
2 Warshaw AL, Fernandez-del Castillo C. Pancreatic carcinoma. *N Engl J Med* 1992; **326**:455–465.
3 Burris H, Storniolo AM. Assessing clinical benefit in the treatment of pancreas cancer: gemcitabine compared to 5-fluorouracil. *Eur J Cancer* 1997; **33 (Suppl 1)**:18–22.
4 Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, *et al.* Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**:2403–2413.
5 Rothenberg ML, Moore MJ, Cripps MC, Andersen JS, Portenoy RK, Burris HA 3rd, *et al.* A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. *Ann Oncol* 1996; **7**:347–353.
6 Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995; **55**:3964–3968.

7 Smith JJ, Derynck R, Korc M. Production of transforming growth factor alpha in human pancreatic cancer cells: evidence for a superagonist autocrine cycle. *Proc Natl Acad Sci U S A* 1987; **84**:7567–7570.

8 Ebert M, Yokoyama M, Kobrin MS, Friess H, Lopez ME, Buchler MW, *et al.* Induction and expression of amphiregulin in human pancreatic cancer. *Cancer Res* 1994; **54**:3959–3962.

9 Kobrin MS, Funatomi H, Friess H, Buchler MW, Stathis P, Korc M. Induction and expression of heparin-binding EGF-like growth factor in human pancreatic cancer. *Biochem Biophys Res Commun* 1994; **202**:1705–1709.

10 Korc M, Chandrasekar B, Yamanaka Y, Friess H, Buchier M, Beger HG. Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increases in the levels of epidermal growth factor and transforming growth factor alpha. *J Clin Invest* 1992; **90**:1352–1360.

11 Wagner M, Cao T, Lopez ME, Hope C, van Nostrand K, Kobrin MS, *et al.* Expression of a truncated EGF receptor is associated with inhibition of pancreatic cancer cell growth and enhanced sensitivity to cisplatinum. *Int J Cancer* 1996; **68**:782–787.

12 Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; **9**:669–676.

13 Perrotte P, Matsumoto T, Inoue K, Kuniyasu H, Eve BY, Hicklin DJ, *et al.* Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 1999; **5**:257–265.

14 Rak J, Filmus J, Kerbel RS. Reciprocal paracrine interactions between tumour cells and endothelial cells: the 'angiogenesis progression' hypothesis. *Eur J Cancer* 1996; **32A**:2438–2450.

15 Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B, *et al.* Neutralizing antibodies against epidermal growth factor and ErbB-2/*neu* receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997; **151**:1523–1530.

16 Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Chester R, Jackson JA, *et al.* ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res* 2002; **62**:4645–4655.

17 Bruns CJ, Harbison MT, Kuniyasu H, Eue I, Fidler IJ. *In vivo* selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. *Neoplasia* 1999; **1**:50–62.

18 Guba M, Cernaianu G, Koehl G, Geissler EK, Jauch KW, Anthuber M. A primary tumor promotes dormancy of solitary tumor cells before inhibiting angiogenesis. *Cancer Res* 2001; **61**:5575–5579.

19 Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, *et al.* Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med* 2002; **8**:128–135.

20 Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev* 1997; **18**:4–25.

21 Plate KH, Breier G, Millauer B, Ullrich A, Risau W. Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. *Cancer Res* 1993; **53**:5822–5827.

22 Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, *et al.* High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 1993; **72**:835–846.

23 Kumar R, Kuniyasu H, Bucana CD, Wilson MR, Fidler IJ. Spatial and temporal expression of angiogenic molecules during tumor growth and progression. *Oncol Res* 1998; **10**:301–311.

24 Ellis LM, Fidler IJ. Angiogenesis and metastasis. *Eur J Cancer* 1996; **32A**:2451–2460.

25 Fidler IJ, Singh RK, Yoneda J, Kumar R, Xu L, Dong Z, *et al.* Critical determinants of neoplastic angiogenesis. *Cancer J* 2000; **6 (Suppl 3)**: 225–236.

26 Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, *et al.* Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 1997; **57**:4593–4599.

27 Lin P, Sankar S, Shan S, Dewhirst MW, Polverini PJ, Quinn TQ, *et al.* Inhibition of tumor growth by targeting tumor endothelium using a soluble vascular endothelial growth factor receptor. *Cell Growth Differ* 1998; **9**: 49–58.

28 Cheng SY, Huang HJ, Nagane M, Ji XD, Wang D, Shih CC, *et al.* Suppression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. *Proc Natl Acad Sci U S A* 1996; **93**:8502–8507.

29 Ramakrishnan S, Olson TA, Bautch VL, Mohanraj D. Vascular endothelial growth factor-toxin conjugate specifically inhibits KDR/flk-1-positive endothelial cell proliferation *in vitro* and angiogenesis *in vivo*. *Cancer Res* 1996; **56**:1324–1330.

30 Millauer B, Longhi MP, Plate KH, Shawver LK, Risau W, Ullrich A, *et al.* Dominant-negative inhibition of Flk-1 suppresses the growth of many tumor types in vivo. *Cancer Res* 1996; **56**:1615–1620.

31 Ikeda N, Adachi M, Taki T, Huang C, Hashida H, Takabayashi A, *et al.* Prognostic significance of angiogenesis in human pancreatic cancer. *Br J Cancer* 1999; **79**:1553–1563.

32 Bruns CJ, Liu W, Davis DW, Shaheen RM, McConkey DJ, Wilson MR, *et al.* Vascular endothelial growth factor is an *in vivo* survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. *Cancer* 2000; **9**:488–499.

33 Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, *et al.* Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res* 2000; **60**:2926–2935.

34 Solorzano CC, Baker CH, Bruns CJ, Killion JJ, Ellis LM, Wood J, *et al.* Inhibition of growth and metastasis of human pancreatic cancer growing in nude mice by PTK 787/ZK222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases. *Cancer Biother Radiopharm* 2001; **16**:359–370.

35 Giannelli G, Azzariti A, Sgarra C, Porcelli L, Antonaci S, Paradiso A. ZD6474 inhibits proliferation and invasion of human hepatocellular carcinoma cells. *Biochem Pharmacol* 2006; **71**:479–485.

36 Wu W, Isobe T, Itasaka S, Shintani T, Langley RR, Onn A, *et al.* ZD6474, a small molecule targeting VEGF and EGF receptor signaling, inhibits lung angiogenesis and metastasis and improves survival in an orthotopic model of non-small cell lung cancer. *Proc Am Assoc Cancer Res* 2004; **45**:1051.

37 Matsumori Y, Goto H, Nakataki E, Kanematsu T, Yano S, Wedge S, *et al.* ZD6474, an inhibitor of vascular endothelial growth factor receptor tyrosine kinase activity, inhibits growth of human non-small cell lung cancer metastases that are resistant to treatment with ZD1839. *Proc Am Assoc Cancer Res* 2003; **44**:9.

38 Heffelfinger S, Gear R, Yan M, Schneider J, LaDow K, Warshawsky D. ZD6474 prevents the formation of ductal hyperplasia, carcinoma *in situ*, and invasive tumors in the DMBA model of rat mammary carcinogenesis. *Proc Am Assoc Cancer Res* 2003; **44**:1130.

39 Wedge SR, Kendrew J, Klinowska T, Ogilvie DJ, Westwood RF, Wadsworth PF, *et al.* The VEGF receptor tyrosine kinase inhibitor ZD6474 is efficacious in a spontaneously metastatic human tumor model. *Clin Cancer Res* 2003; **7**:3653S [abstract 4].

40 Ciardiello F, Bianco R, Caputo R, Caputo R, Damiano V, Troiani T, *et al.* Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. *Clin Cancer Res* 2004; **10**:784–793.

41 Ciardiello F, Bianco R, Caputo R, Caputo R, Damiano V, Troiani T, *et al.* Antitumor activity of ZD6474, a small molecule VEGF receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to EGF receptor-targeted drugs. *Proc Am Soc Clin Oncol* 2003; **22**:205.

42 Bruns CJ, Shrader M, Harbison MT, Portera C, Solorzano CC, Hicklin DJ, *et al.* Effect of the VEGF-R2 antibody, DC101, + Gemcitabine on growth, metastasis, and angiogenesis of human pancreatic cancer growing orthotopically in nude mice. *Int J Cancer* 2002; **102**:101–108.

43 Baker CH, Solorzano CC, Fidler IJ. Blockade of vascular endothelial growth factor receptor and epidermal growth factor receptor signaling for therapy of metastatic human pancreatic cancer. *Cancer Res* 2002; **62**:1996–2003.

44 Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, *et al.* PTK787/ ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res* 2000; **60**:2178–2189.

45 Shimizu K, Kubo H, Yamaguchi K, Kawashima K, Ueda Y, Matsuo K, *et al.* Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer. *Cancer Sci* 2004; **95**:328–333.