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Antiangiogenic versus cytotoxic therapeutic approaches to human pancreas cancer: an experimental study with a vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor and gemcitabine

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Abstract

Pancreatic adenocarcinoma is a leading cause of cancer death in the United States and represents a challenging chemotherapeutic problem. The pharmacological control of angiogenesis might represent a novel approach to the management of pancreas cancer, since the pathological development of vascular supply is a critical step for tumor growth and may affect its prognosis. In order to test this hypothesis, SU5416 ([3-(3,5-dimethyl-1*H*-pyrrol-2-ylmethylene)-1,3-dihydro-indol-2-one]) a selective inhibitor of the vascular endothelial growth factor receptor-2 tyrosine kinase, and gemcitabine (2′, 2′-difluorodeoxycytidine) were tested on endothelial (HUVEC) and pancreatic tumor cells (MIA PaCa-2) in vitro and in vivo alone and in simultaneous association. SU5416 inhibited HUVEC cells stimulated to proliferate by vascular endothelial growth factor but not MIA PaCa-2 cells; the drug concentration that decreased cell growth by 50% (IC_{50}) was 0.14 μM. Furthermore, SU5416 reduced the development of microvessels from placental explants (IC_{50}, 0.23 μM). Gemcitabine inhibited the growth of both HUVEC and MIA PaCa-2 cells with an IC_{50} of 0.08 and 0.1 μM, respectively. A synergistic effect (combination index <1 and dose reduction index >1) on anti-proliferative and pro-apoptotic activity was calculated with the simultaneous combination of the two drugs on endothelial cells. A marked in vivo antitumor effect on MIA PaCa-2 xenografts was observed with SU5416 at a protracted schedules, as well as with gemcitabine; furthermore, the combination between the two drugs resulted in an almost complete suppression of tumor growth and relapse. In conclusion, the present results provide the evidence of an effective anti-endothelial/antitumor activity of protracted administration of SU5416 on human pancreas cancer xenografts, which is comparable with the one obtained by gemcitabine; moreover, the synergistic combination between these drugs on endothelial cells and the promising association in pancreatic cancer xenografts could be used in future studies and translated into the clinical setting.
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Keywords: SU5416; Gemcitabine; Pancreas cancer cell; Endothelial cell; VEGF; Angiogenesis

1. Introduction

Pancreatic adenocarcinoma is a leading cause of cancer death in the United States, with approximately 28,000 deaths annually (Abbruzzese, 2002) and still represents a challenging therapeutic problem because it is widely recognized to be resistant to surgery, radiotherapy, and chemotherapy; furthermore, no major advances in the treatment of this disease were obtained during recent years (McKenna and Eatock, 2003). The chemotherapeutic approach to pancreas cancer almost entirely relied on the use of fluoropyrimidines (e.g. 5-

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fluorouracil); more recently, the cytosine arabinoside analogue gemcitabine (2′, 2′-difluorodeoxycytidine) alone or in association with other antineoplastic drugs, such as docetaxel and irinotecan, displayed enhanced clinical activity over 5-fluorouracil, although survival was marginally increased (Jacobs, 2002; Berlin and Rothenberg, 2003).

The pharmacological control of angiogenesis might represent a novel approach to the management of pancreas cancer, since the pathological development of vascular supply is a critical step for tumor growth and may affect its prognosis (Wang et al., 2003; Kuehn et al., 1999; Ikeda et al., 1999). Moreover, Schuch et al. (2002) demonstrated that pancreatic tumor growth is dependent on its ability to increase the angiogenic stimulus—such as vascular endothelial growth factor (VEGF)—or to reduce the amounts of endogenous antiangiogenic factors (such as endostatin). Among the various pro-angiogenic molecules VEGF, a homodimeric glycoprotein, plays a prominent role in vessel development (Fujimoto et al., 1998), local progression (Itakura et al., 1997), metastatic spread and poor prognosis of pancreatic adenocarcinoma (Niedergethmann et al., 2002). Many pancreatic cell lines as MIAPaCa-2, Capan-1 and AsPC-1 show an over-expression of VEGF in vitro (Itakura et al., 2000). Furthermore, high-affinity VEGF receptors flt-1 (VEGFR-1) and flk-1/kinase domain receptor (VEGFR-2) were found on the endothelial cells of blood vessels of pancreatic cancer in vivo (Brown et al., 1993), influencing the prognosis of patients (Buchler et al., 2002). The VEGF receptor family is characterized by the presence of a tyrosine kinase intracellular domain and VEGFR-2 is considered the major receptor transducing the mitogen and chemotactic effect of VEGF into endothelial cells (Cross et al., 2003).

The importance of VEGFR-2 in pancreatic cancer angiogenesis has suggested that blocking this receptor could be a useful therapeutic target to inhibit neovascularization and tumour progression. Indeed, recent experimental approaches with an anti-VEGFR-2 antibody such as DC101 (Bruns et al., 2002) or small molecules targeting the VEGF tyrosine kinase (Baker et al., 2002; Solorzano et al., 2001) alone or in combination with chemotherapeutic drugs (Bruns et al., 2002; Baker et al., 2002), have shown promising results in vivo.

SU5416 ([3-(3,5-dimethyl-1*H*-pyrrol-2-ylmethylene)-1,3-dihydro-indol-2-one]) is a selective inhibitor of the VEGFR-2 tyrosine kinase (Fong et al., 1999); the drug has been successfully used alone in vivo to reduce the growth of xenotransplanted tumors such as melanoma, lung, prostatic and mammary carcinoma, glioma and fibrosarcoma (Fong et al., 1999) and of colon cancer liver metastasis in mice (Shaheen et al., 1999). Combination studies have been performed with low-dose endostatin (Abdollahi et al., 2003), SU6668 (Erber et al., 2004) or fractionated radiotherapy (Trinh et al., 2003; Lund et al., 2003) to enhance the antiangiogenic/antitumor activity of the compound. Furthermore, SU5416 has been used in several clinical studies involving patients suffering of renal cell carcinomas (Kuenen et al., 2003a), acute myeloid leukemias (Fiedler et al., 2003), retinal hemangioblastomas (Girmens et al., 2003), refractory myeloproliferative diseases (Giles et al., 2003) and metastatic colorectal cancers (DePrimo et al., 2003) in order to establish its safety and efficacy profile and find possible surrogate markers for treatment optimization.

To provide a rationale for improving the therapeutic efficacy of gemcitabine-based combination schedules in pancreatic cancer, this study was designed to determine in various experimental settings the activity of a VEGFR-2 inhibitor alone such as SU5416 and in combination with gemcitabine on cancer cell growth and angiogenesis in vitro as well as the antitumor effects in vivo.

2. Materials and methods

2.1. Reagents and animals

Recombinant human vascular endothelial growth factor (rhVEGF) and recombinant human epidermal growth factor (rhEGF) were from PeproTechEC (London, UK). Cell culture Medium 199 and Dulbecco's Minimum Essential Medium (DMEM), fetal bovine serum, horse serum, L-glutamine, penicillin, streptomycin, gentamycin were from Gibco (Gaithersburg, MD, USA). Type A gelatin from porcine skin, supplements and all other chemicals not listed in this section were obtained from Sigma (St. Louis, MO, USA). Plastics for cell culture were supplied by Costar (Cambridge, MA, USA).

SU5416 and gemcitabine were generous gifts from SUGEN (South San Francisco, CA, USA) and Ely Lilly and Company (Indianapolis, IN, USA), respectively; gemcitabine was dissolved in sterile distilled water and stored at −20 °C, whereas SU5416 was dissolved in 100% dimethyl sulfoxide (DMSO) for in vitro use (Fong et al., 1999) and in a solution of 99% polyethylene glycol (PEG)-300 (w/v) and 1% Tween 80 for in vivo studies (Shaheen et al., 1999).

The CD nu/nu male mice, weighing 20–25 g, were supplied by Charles River (Milan, Italy) and were allowed unrestricted access to food and tap water. Their care and handling were in accordance with all applicable regulations on animal experimentation.

2.2. Cell culture conditions

Human umbilical vein endothelial cells (HUVEC) were obtained and prepared as previously described (Bussolino et al., 1992). Briefly, the luminal surface of placenta veins was incubated for 10 min at 37 °C with 0.25% collagenase type IA in phosphate-buffered saline (PBS). Cells were centrifuged, washed in PBS and cultured in tissue flasks precoated with 1% gelatin in Medium 199, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 IU/ml), streptomycin (50 μg/ml), amphotericin B (2.5 μg/

ml), L-glutamine (2 mM), rhEGF (10 ng/ml) and heparin (10 U/ml).

The human pancreatic cancer cell line MIA PaCa-2 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MIA PaCa-2 cells, established and characterized by Yunis et al. (1977), were maintained in DMEM medium, supplemented with 10% fetal bovine serum, 2.5% horse serum, penicillin (50 IU/ml), streptomycin (50 μg/ml) and L-glutamine (2 mM). HUVEC and MIA PaCa-2 cells were routinely grown in 75 cm^2 tissue culture flasks and kept in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were harvested with a solution of 0.25% trypsin–0.03% EDTA when they were in log phase of growth, and maintained at the above-described culture conditions for all experiments.

2.3. Cytotoxicity assay and apoptosis measurements

In vitro chemosensitivity testing was performed on HUVEC (6×10^4 cells/well) and MIA PaCa-2 cells (2×10^4 cells/well) plated in 6-well sterile plastic plates and allowed to attach overnight. Cells were treated with SU5416 and gemcitabine (0.01–100 μM) or with their vehicles for 30 min, then media were replaced with drug-free medium and cells cultured for additional 96 h; in separate experiments, HUVEC and MIA PaCa-2 cells received SU5416 (0.01–1 μM) in combination with rhVEGF 10 ng/ml for 72 h. At the end of the treatment, the cells were harvested with trypsin/EDTA, and counted with an hemocytometer; the growth of treated cells was expressed as a percentage of control cultures (vehicle alone). The DMSO concentration in control wells was the same used to dilute the highest dose of SU5416 in a similar experiment. The concentration of drugs that decreased cell count by 50% (IC_{50}) as compared to controls was calculated by nonlinear fitting of experimental data.

In order to quantify the apoptosis induced by the treatments to endothelial cells, HUVEC cells (supplemented with rhVEGF 10 ng/ml) were treated for 72 h with SU5416 100 nM, gemcitabine 100 nM, SU5416 100 nM plus gemcitabine 100 nM and vehicle alone. At the end of the experiment, cells were collected and the Cell Death Detection Enzyme-Linked ImmunoSorbent Assay (ELISA) Plus kit (F. Hoffmann-La Roche, Basel, Switzerland) was used. All the absorbance values were plotted as a percentage of apoptosis relative to control cells (vehicle only), which were labeled as 100%. All experiments were repeated three times with at least two replicates per sample.

2.4. In vitro assessment of synergism between SU5416 and gemcitabine on endothelial cells

SU5416 combined with gemcitabine was tested on endothelial cells supplemented with rhVEGF 10 ng/ml with the treatment schedule at a fixed molar concentration ratio of 1:1, as follows: simultaneous exposure of SU5416 (0.01–1 μM) plus gemcitabine (0.01–1 μM) for 72 h. To evaluate the level of interaction (synergistic, additive or antagonist) between gemcitabine and SU5416, the method proposed by Chou et al. (1993) was followed. Briefly, synergism or antagonism for SU5416 plus gemcitabine was calculated on the basis of the multiple drug-effect equation, and quantitated by the combination index (CI) where CI<1, CI=1, and CI>1 indicates synergism, additive effect, and antagonism, respectively. Based on the classic isobologram for mutually exclusive effects, the CI value was calculated as:

$$CI = \left[(D)_1/(D_x)_1\right] + \left[(D)_2/(D_x)_2\right]$$

At 50% inhibition level, $(D_x)_1$ and $(D_x)_2$ are the concentrations of SU5416 and gemcitabine, respectively, that induce a 50% inhibition of cell growth; $(D)_1$ and $(D)_2$ are the concentrations of SU5416 and gemcitabine in combination that also inhibits cell growth by 50% (iso-effective as compared with the single drugs alone). The dose-reduction index (DRI) defines the degree of dose reduction that is possible in a combination for a given degree of effect as compared with the concentration of each drug alone:

$$(DRI)_1 = (D_x)_1/(D)_1 \text{ and } (DRI)_2 = (D_x)_2/(D)_2$$

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of VEGFR-2 expression

Total RNA was extracted from MIA PaCa-2 cells using Trizol (Life Technologies, Houston, TX). Two pairs of oligomers were synthesized based on the reported sequence of human VEGFR-2 (5′ -ATG GTG TAA CCC GGA GTG ACC A-3′ and 5′ -GTT GGC GCA CTC TTC CTC CAA-3′ , forward and reverse primers, respectively) (Meister et al., 1999) and β-actin as house-keeping gene. RT-PCR was performed using the cDNAs obtained by reverse transcription of RNA extracted from tumor cells, as templates, and the oligomers as primers. The thermal cycle profile for cDNA amplification was the following: initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation (1 min at 94 °C), annealing (1 min at 50 °C), synthesis (1 min at 72 °C) and final extension for 10 min at 72 °C. After the reaction, PCR products were separated by 2% agarose gel electrophoresis in Tris acetate-EDTA buffer and visualized by ethidium bromide staining.

2.6. In vitro cultures of human placental vessels

The use of explants from human placenta and from umbilical cord was approved by the Ethics Committee of Pisa University Hospital. A written informed consent on the procedures and aims of the study was obtained from each placenta donor for compliance with regulations

concerning the use of human tissues. The experimental procedure described by Bocci et al. (2001) was followed. Briefly, superficial blood vessels (diameter, 1 to 1.5 mm) were quickly excised from the apical surface of human placentas and cut in 1-mm fragments. Vessel fragments were included in a fibrin gel, obtained by mixing fibrinogen (1 ml/well of a 3 mg/ml solution in Medium 199) with 30 μl of thrombin (50 NIH U/ml in 0.15 M NaCl), into each well of 24-well culture plates. After gel formation, 1 ml/well of Medium 199, supplemented with 10% of heat-inactivated fetal bovine serum, 0.1% ε-aminocaproic acid, 2 mM L-glutamine and antibiotics (streptomycin 50 μg/ml, penicillin 50 IU/ml and amphotericin B 2.5 μg/ml), was added. Vessel fragments were treated every other day with SU5416 (0.01–1 μM) or with vehicle alone and cultured at 37 °C in 95% air/5% CO_2 in a humidified environment for 28 days. Vessel explants were photographed on day 28 with a phase-contrast Leitz MD IL microscope (Leica, Heerbrugg, Switzerland) and pictures of at least nine explants per drug level were subjected to image analysis. Pictures of vessel explants were digitized in a 512×512-pixel matrix and processed with the true colour image analysis software package KS 300 v.1.2 (Kontron Elektronik, Eching, Germany) for interactive quantification and data collection. Geometric calibrations were set with a sample of known dimensions and a gray-scale analysis was performed to measure the density of the image. The mean gray level of the endothelial sprouting area was measured and the sprouting index was defined as follows:

$$\text{sprouting index} = [(\text{sprouting area}/\text{mean gray level of sprouting area}) / \text{perimeter of fragments}] \times 100\,.$$

Results were calculated as percentage of control sprouting index and expressed as mean±S.E.

2.7. In vivo studies

MIA PaCa-2 cell viability was assessed by trypan blue dye exclusion and on day 0, $1.3\times10^6\pm5\%$ cells/mouse were inoculated subcutaneously between the scapulae in 0.2 ml/mouse of culture medium without fetal bovine serum using an insulin syringe with a 0.5×16-mm needle. Animals were monitored until appearance of a subcutaneous tumor that was measured twice weekly in two perpendicular directions using calipers. Tumor volume (mm^3) was defined as follows: $[(w_1\times w_2\times w_2)\times(\pi/6)]$, where w_1 and w_2 were the largest and the smallest tumor diameter (mm), respectively (Prewett et al., 1999). In order to mimic a possible antiangiogenic/chemotherapeutic treatment soon after surgery—with minimal residual disease—, on day 1, about 24 h from cell inoculum, SU5416, gemcitabine or their combination were administered intraperitoneally as follows: (1) SU5416 twice weekly at the dose of 50 mg/kg for 14 days or 25 mg/kg for 28 days; (2) gemcitabine 120 mg/kg four times at 3-day intervals as previously described (Braakhuis et al., 1995); (3) combination treatment of SU5416 at each of the above schedules and gemcitabine. The control group was injected i.p. with vehicle alone. The experimental period ended 14 days after the last injection of SU5416. Mice were sacrificed by an anesthetic overdose and tumors were excised, measured and sampled for immunohistochemistry to detect the protein expression of VEGF in tumour cells and of VEGFR-2 in tumour blood vessels.

2.8. Immunohistochemistry

Tissue samples were fixed in 10% phosphate-buffered formaldehyde for 12–24 h and embedded in paraffin. Tissue sections (4 μm thick) were immunostained with the streptavidin–peroxidase technique, using the automated Ventana's NexES IHC Staining System. The primary antibodies for antigen detection were mouse anti-human VEGF (Neomarkers, Fremont, CA; 1:100 dilution) and rabbit anti-mouse flk-1/kinase domain receptor (VEGFR-2; Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution). VEGF antigen unmasking was obtained by microwaving tissue specimens in citrate buffer, pH 7.3 (three cycles of 5 min each, 750 W), while tissue sections were boiled in a pressure cooker for 5 min in citrate buffer, pH 7.3, for VEGFR-2 unmasking. Immunoreactivity was evidenced by a brown staining in the cytoplasm of neoplastic cells (VEGF) and endothelial cells of tumor vessels (VEGFR-2).

2.9. Analysis of data

Data are expressed as a mean value±S.E. Statistical analysis was performed by the two-tail unpaired Student's *t*-test; *P* values lower than 0.05 were considered significant.

3. Results

3.1. Inhibition of cell growth by SU5416, gemcitabine and their simultaneous combination

Gemcitabine inhibited cell growth of HUVEC and MIA PaCa-2 cell line in a concentration-dependent manner (Fig. 1), and the IC_{50} values were 0.08 and 0.10 μM, respectively; on the contrary, SU5416 did not affect the proliferation of either cell line (IC_{50}>100 μM; Fig. 1). In this experimental setting (30 min of exposure), high doses of SU5416 (100 μM) did not produce any effect on proliferation, whereas, as expected, the antineoplastic cytotoxic drug gemcitabine greatly inhibited proliferation, due to the well-known inhibition of DNA synthesis and function. In contrast, SU5416 prolonged exposures (72 h) at lower concentrations were able to inhibit the growth of

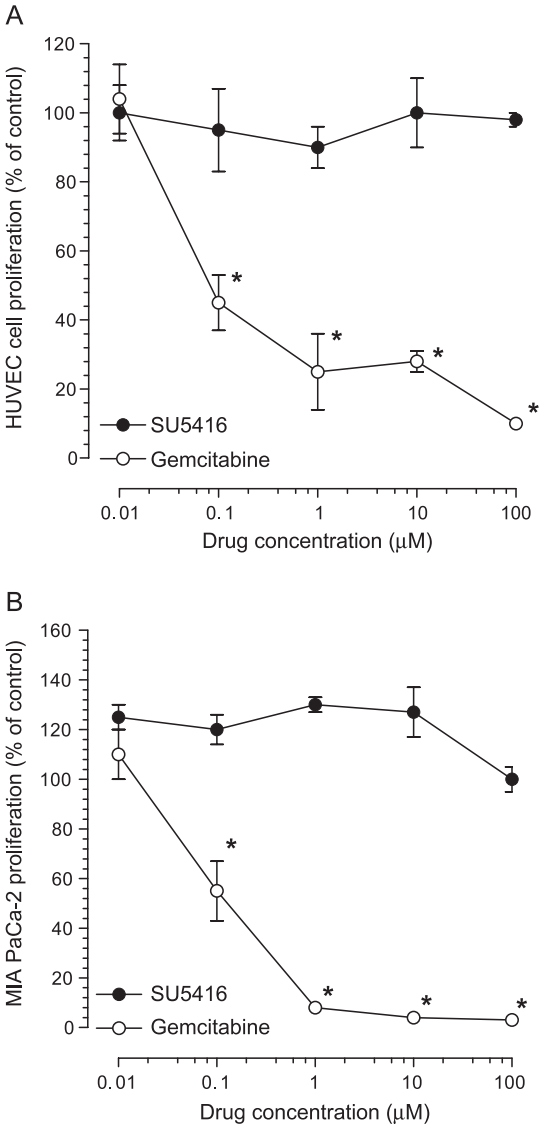

Fig. 1. Effect of SU5416 and gemcitabine on proliferation of human HUVEC (A) and of MIA PaCa-2 (B) cells. Results are the means of three independent experiments±S.E. (vertical bars); *P<0.05 vs. controls.

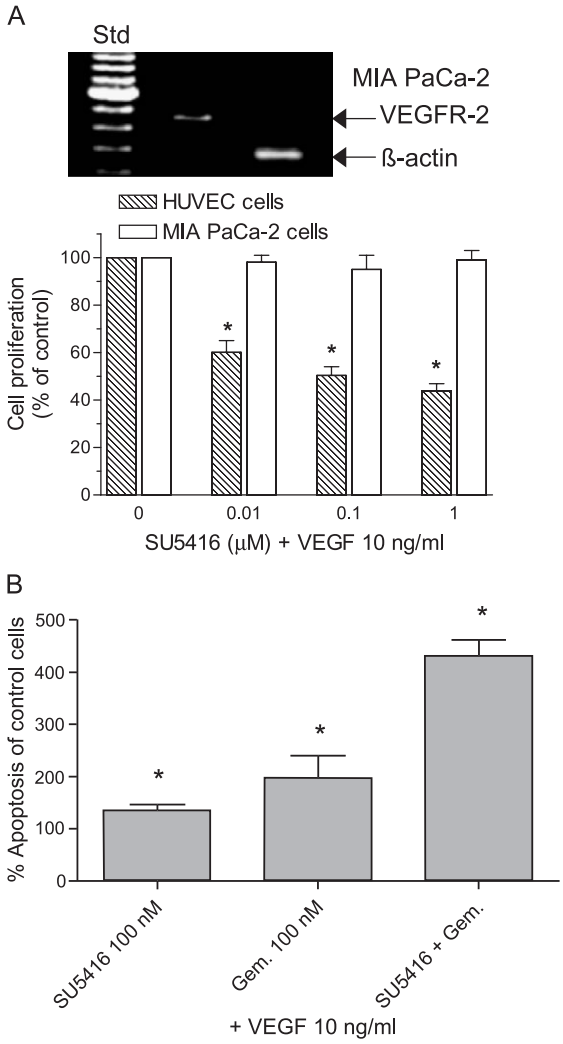

Fig. 2. (A) Expression of VEGFR-2 by MIA PaCa-2 cells, as detected by RT-PCR (upper), and effect of SU5416 on HUVEC and MIA PaCa-2 cell proliferation stimulated with VEGF 10 ng/ml. (B) Induction of cell apoptosis after 72-h treatment with SU5416 100 nM, gemcitabine 100 nM and their simultaneous combination on HUVECs stimulated with VEGF 10 ng/ml. Results are the means of three independent experiments±S.E. (columns and vertical bars, respectively); *P<0.05 vs. controls.

HUVEC cells if they were stimulated to proliferate by VEGF 10 ng/ml in the culture medium (Fig. 2A), as demonstrated by an IC_{50} value of 0.14 μM. Finally, the proliferation of MIA PaCa-2 cells incubated with VEGF 10 ng/ml was not enhanced, despite the detection of a faint VEGFR-2 amplification fragment on PCR analysis, and SU5416 up to 1 μM did not affect their growth (Fig. 2A). As shown in Fig. 2B, the Cell Death ELISA assay also revealed a markedly higher levels of apoptosis in the SU5416 and gemcitabine-treated endothelial cells when compared to controls, after 72 h of 100 nM treatment in media supplemented with VEGF 10 ng/ml. Furthermore, the simultaneous combination schedule of the two drugs greatly enhanced the pro-apoptotic effects on endothelial cells (Fig. 2B). Indeed, simultaneous exposure of endothelial cells to SU5416 and gemcitabine for 72 h showed synergism at effect levels of 35%, 50% and 65% inhibition of cell proliferation (Table 1). Synergism corresponding to CI<1 always yielded a favourable dose-reduction index (DRI>1) for both drugs (Tables 1 and 2). The dose-reduction index values at IC_{35}, IC_{50}, and IC_{65} are reported in Table 2.

Table 1
Combination index (CI) values for the drug combination at 35%, 50% and 65% level of inhibition of HUVEC cell growth

Drug combination	CI values (mean±S.E.)		
	35%	50%	65%
SU5416+gemcitabine (molar ratio 1:1)	0.008±0.003	0.043±0.01	0.456±0.1

Table 2
Dose-reduction index (DRI) values for the combination at 35%, 50% and 65% level of inhibition of HUVEC cell growth

Drug combination	DRI values (mean±S.E.)					
	35%		50%		65%	
	SU5416	Gemcitabine	SU5416	Gemcitabine	SU5416	Gemcitabine
SU5416+gemcitabine (molar ratio 1:1)	304±29	224±19	26±3	246±32	2.2±0.4	271±24

3.2. Inhibition of endothelial cell culture from placental blood vessel by SU5416

The endothelial cell sprouting within the fibrin matrix was characterized by the radial growth of numerous microvessels around the placental explants. Maximal development of the three-dimensional microvascular network occurred during the third week of culture and reached the plateau at day 28 of culture (Fig. 3A). Endothelial cells organized radially to form microvessels that underwent continuous remodelling. SU5416 markedly inhibited the proliferation of endothelial cells as well as the production of vascular structures by inducing a decrease in the density and length of vascular sprouts (Fig. 3B). The sprouting index was concentration-dependently inhibited by SU5416 (IC_{50}: 0.23 μM; $P<0.05$ vs. controls at day 28, Fig. 4).

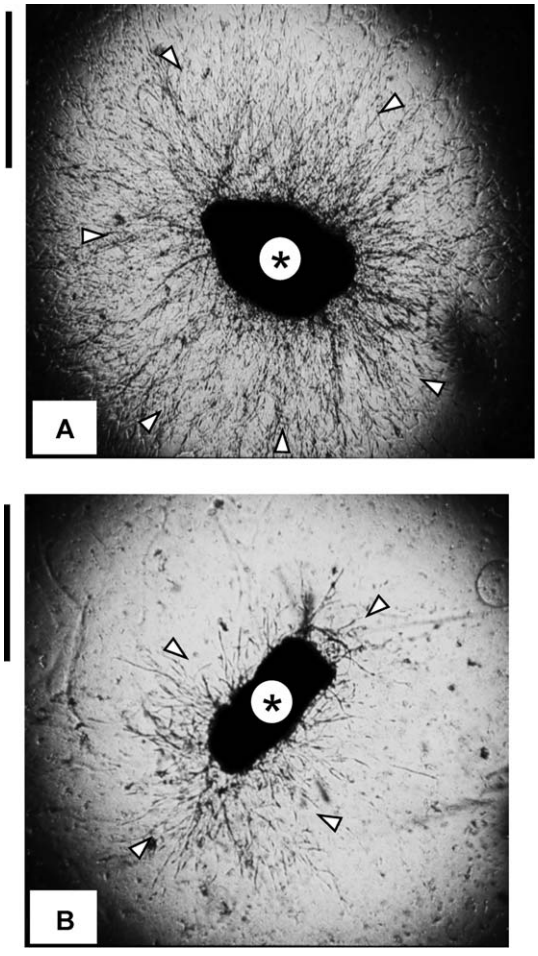

Fig. 3. Microscopic picture of a representative sample of fibrin-embedded placental explant that shows microvessel structures extending radially (A, arrowheads). SU5416 (1 μM for 28 days) markedly reduced the proliferation and migration of endothelial cells (B, arrowheads). *, placental vessel explant; bars, 2 mm.

3.3. Inhibition of tumor growth in vivo by SU5416 and gemcitabine

Tumor xenografts were detectable within the first week from cell injection; in the control group, the mean volume of tumor nodules was 652.9 mm^3 at the end of the first study (day 28), whereas at the end of the second experiment (day 42) the mean volume was 944 mm^3. A significant in vivo antitumor effect of SU5416 was detected with both schedules (Fig. 5); however, the administration of the higher dose of SU5416 for a shorter period of time (50 mg/kg for 14 days) proved to be less effective than the lower dose for a longer period of time (25 mg/kg for 28 days; −69.4% vs. −98.4%, respectively, at the end of experiments; Fig. 5). The tumor growth inhibition obtained with gemcitabine 120 mg/kg four times at 3-day intervals was similar at days 28 and 42 (−88.4% and −87%, respectively, vs. controls). The combination of SU5416 50 mg/kg for 14 days or 25 mg/kg for 28 days and gemcitabine resulted in an almost complete regression of tumor volumes (−95% at day 28 and −100% at day 42; Fig. 5). Interestingly, both the schedules showed tumor relapses in all the treated groups at the end of the experiments, with the significant exception of the 28-day combination treatment (Fig. 5A and B, top left inset).

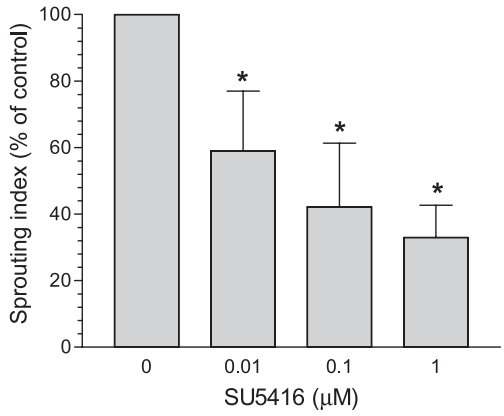

Fig. 4. Inhibition of endothelial cell sprouting by SU5416. Results are the means of sprouting index from three independent experiments±S.E. (vertical bars); *$P<0.05$ vs. controls.

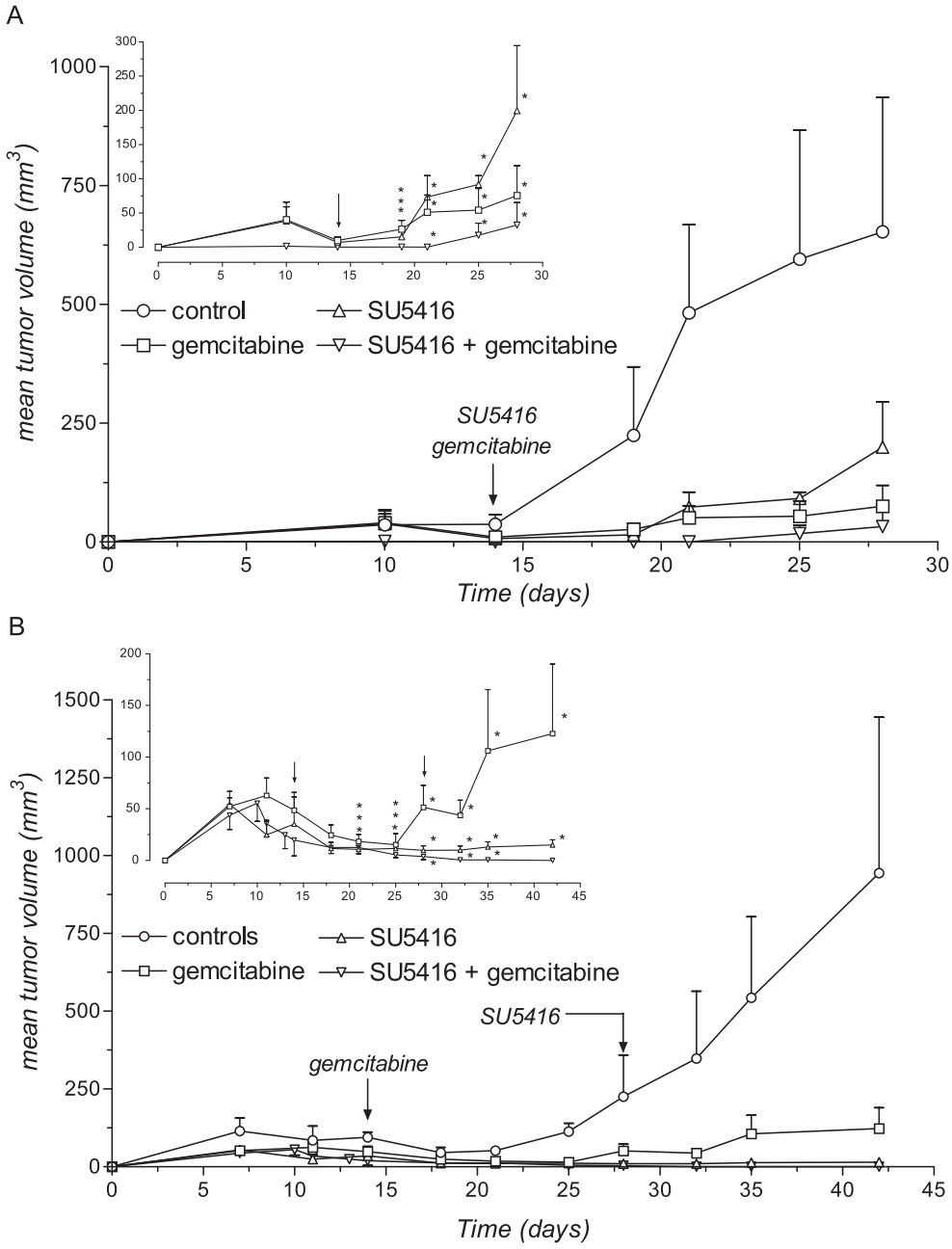

Fig. 5. Inhibition of in vivo growth of MIA PaCa-2 tumours in animals treated with SU5416 50 mg/kg for 14 days (A) or 25 mg/kg for 28 days (B), gemcitabine 120 mg/kg and their combination. The inset (top left) has magnified axes for the tumour volume of treated groups only. Means±S.E. (vertical bars); *P<0.05 vs. controls; ↓ end of treatments.

SU5416 treatments were tolerated well, and no difference in animal behavior or weight was found between groups.

3.4. Immunohistochemistry

The subcutaneous injection of MIA PaCa-2 pancreas cancer cells produced a tumor whose histological picture, after staining with hematoxylin and eosin, was consistent with adenocarcinoma (data not shown). Tumor xenografts showed a diffuse, easily detectable immunoreactivity to the anti-VEGF antibody within cancer cells and a strong VEGFR-2 immunoreactivity localized in endothelial cells inside the tumor mass (Fig. 6).

4. Discussion

Among the numerous compounds recently used in the preclinical and clinical studies against VEGF and VEGF receptors (Ferrara et al., 2003), SU5416 is a small lipophilic synthetic molecule that selectively inhibits tyrosine kinase activity of VEGFR-2 (Fong et al., 1999; Mendel et al.,

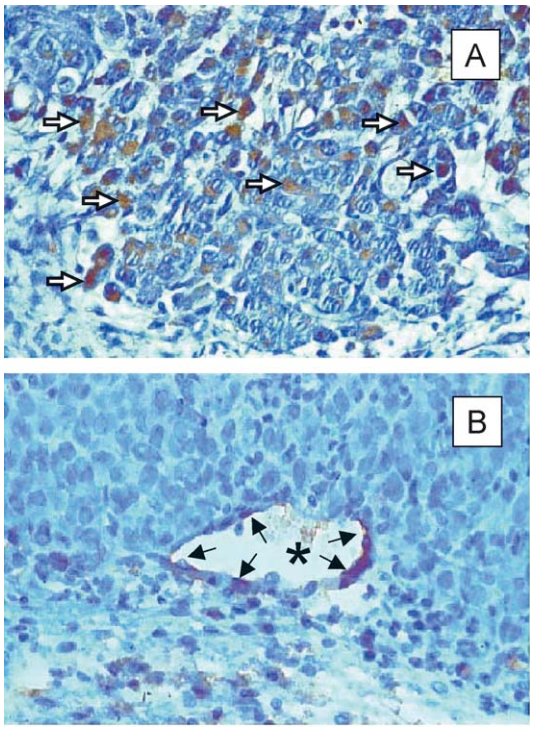

Fig. 6. Immunohistochemistry of VEGF (A) in MIA PaCa-2 xenografts and VEGFR-2 (B) in endothelial cells of nude mice. Arrowheads, positive-staining cells; *, blood vessel.

2000), the major receptor transducing the mitogenic and survival signal of VEGF into endothelial cells (Cross et al., 2003). The in vitro experiments of this study showed that SU5416 was able to inhibit the growth-promoting and survival activity of VEGF on endothelial cells—not on pancreatic cancer cells—and to block the complex process of microvessels formation in protracted, long-term (28 days) treatments in the human placental 3D assay. Furthermore, SU5416 synergistically interacted with gemcitabine both in vitro and in vivo allowing a rational and possible dose adjustment in long-term treatments of pancreatic cancers and reducing the toxic impact of the therapy. Among recent clinical studies using SU5416 alone or in combination with chemotherapeutic drugs, efficacy and toxicity data on SU5416/gemcitabine schedules are still not available in pancreatic cancer. However, recently Kuenen et al. (2002), performing a dose-finding and pharmacokinetic study of the combination cisplatin, gemcitabine and SU5416 in patients with various solid tumours, described a deep venous thrombosis in patient no. 8 who had pancreatic carcinoma. The authors seemed to suggest a major role of cisplatin in the development of a prothrombotic state (Kuenen et al., 2003b); in contrast, no increased incidence of thromboembolic events has been observed with SU5416 in combination with other cytotoxic agents such as 5-fluorouracil, irinotecan and doxorubicin (Kuenen et al., 2003b).

MIA PaCa-2 pancreatic cancer cells carry a mutation in K-ras oncogene and were chosen because of their ability to produce and secrete VEGF in vitro (Itakura et al., 1997, 2000) and in vivo when transplanted in nude mice, as shown in this study by immunohistochemistry. SU5416 did not affect the proliferation of MIA PaCa-2 cells, even in the presence of exogenous VEGF, possibly because of the absence of functional VEGFR-2 on tumor cell surface. This finding translates in vivo, where tumor growth inhibition by SU5416 most likely results from inhibition of blood vessel growth upon stimulation of endothelial cells by VEGF secreted by MIA PaCa-2, rather than a direct effect on cancer cells. Furthermore, the deoxycytidine analogue gemcitabine showed a marked antiproliferative effect as a result of the cytotoxicity of the drug toward proliferating cells, including endothelial and pancreatic cancer cells (Kerbel et al., 2000; Ng et al., 2000).

In order to mimic the VEGF-induced microenvironment, a three-dimensional fibrin gel culture was used in this study to investigate the antiangiogenic effect of long-term (28 days) SU5416, because of the evidence that VEGF stimulates the migration of the endothelial cells within the extracellular matrix (Qu et al., 1995). Indeed, VEGF increases the permeability of blood vessels, leading to the extravasation of plasma fibrinogen and its clotting (Nagy et al., 1989). The extravascular fibrin provides a stroma that serves as a substrate for endothelial cell migration and growth of new blood vessels (Nagy et al., 1995). Furthermore, the ex vivo model of human placental vessel explants is characterized by autocrine secretion of VEGF by endothelial cells (Bocci et al., 2001) and allows a long-term treatment to test the activity of antiangiogenic drugs. Indeed, a 28-day administration of SU5416 inhibited the proliferation of endothelial cells in a concentration-dependent manner; the inhibition of microvessel formation in fibrin gel suggests that a long-lasting treatment with SU5416 provides an effective suppression of VEGF-dependent tumor microvascular growth.

Human tumor xenografts—even non-metastatic ectopic/subcutaneous “primary” tumor transplants—can be remarkably predictive of cytotoxic chemotherapeutic drugs that have activity in humans, when the drugs are tested in mice using pharmacokinetically clinically equivalent or “rational” drug doses (Kerbel, 2003). However, what may be different with clinical activity is the magnitude of the benefit observed in mice, both in terms of the degree of tumor responses and overall survival (Kerbel, 2003). Although with the limitations of the above-mentioned ectopic xenografts and the precocious beginning of the treatments in order to mimic a post-surgery condition, our in vivo studies with SU5416 provided evidence of a marked inhibition on tumor growth, consistent with the production of VEGF by tumor xenografts and expression of VEGFR-2 by endothelial cells of tumor stroma, especially with the low-dose, long-term schedule (25 mg/kg for 28 days) in comparison with the shorter course (14 days) of SU5416 at 50 mg/kg. This result is consistent with the hypothesis that angiogenesis inhibitors need to be administered for longer periods than cytotoxic agents (Kerbel and Folkman, 2002) and provides further evidence of the effectiveness of

blocking VEGF signaling via the inactivation of VEGFR-2 as a valid therapeutic strategy for inhibiting tumor growth and angiogenesis, as also reported by Bruns et al. (2002) using a specific anti-VEGFR-2 monoclonal antibody.

Furthermore, the in vitro experimental synergistic interaction between SU5416 and gemcitabine on proliferative and pro-apoptotic activity of the endothelial cells well correlates with the in vivo data. Indeed, the association between antiangiogenic therapy with SU5416 and conventional chemotherapy with gemcitabine resulted in an almost complete regression of MIA PaCa-2 tumor growth; this effect may be the result of three major factors: (i) direct effect of SU5416 on endothelial cells; (ii) direct effect of gemcitabine on tumor cells and endothelial cells, and (iii) prevention of the VEGF-dependent rescue of endothelial cell damage produced by gemcitabine with SU5416 since VEGF is a survival factor for endothelial cells (Ferrara et al., 2003). Moreover, the low-dose, long-term combination schedule, in addition to the antitumor efficacy, may have the advantage of improved tolerability due to a rational reduction of doses of both drugs for their synergistic activity. Recently, a similar combination approach was also successfully used in xenotransplants of human pancreatic cancer cells in nude mice with the DC101 anti-VEGF receptor-2 antibody associated with gemcitabine (Bruns et al., 2002).

In conclusion, the present results provide the evidence of an effective anti-endothelial/antitumor activity of protracted administration of SU5416 on human pancreas cancer xenografts, which is comparable with the one obtained by gemcitabine; moreover, the synergistic combination between these drugs on endothelial cells and the promising association in pancreatic cancer xenografts could be used in future studies and may be translated into the clinical setting for the treatment of advanced pancreatic carcinoma. However, since SU5416 is not longer used in the clinic (except for finishing trials and experimental purposes), compounds with similar mechanistic effects—such as PTK787 (Klem, 2003)—may be used for further clinical testing.

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