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Inhibition of insulin-like growth factor-I receptor (IGF-IR) using NVP-AEW541, a small molecule kinase inhibitor, reduces orthotopic pancreatic cancer growth and angiogenesis

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ABSTRACT

The insulin-like growth factor-I receptor (IGF-IR) is frequently overexpressed and constitutively activated in pancreatic cancer, thus representing a promising target for therapy. We investigated the impact of a novel inhibitor of IGF-IR (NVP-AEW541) on signalling and growth of pancreatic cancer. Human pancreatic cancer cells and endothelial cells were employed, and effects of NVP-AEW541 on signalling pathways investigated by Western blotting. NVP-AEW541 diminished the activation of IGF-IR, IRS-1, Erk, Akt and STAT3. Furthermore, NVP-AEW541 reduced cancer cell proliferation and abrogated migratory effects of IGF-I. NVP-AEW541 elicited a direct effect on endothelial cells in terms of reducing endothelial cell migration. *In vivo*, treatment of mice with NVP-AEW541 significantly reduced orthotopic pancreatic tumour growth, vascularisation, and VEGF expression. Interestingly, NVP-AEW541 lowered serum levels of IGF-binding-protein-3 (IGFBP-3). In conclusion, the IGF-IR inhibitor NVP-AEW541 effectively disrupts IGF-I signalling and reduces pancreatic tumour growth. Hence, blocking IGF-IR could prove valuable for targeted therapy of pancreatic cancer.

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1. Introduction

The insulin-like growth factor-I receptor (IGF-IR) system has emerged as an interesting target for cancer therapy, as it represents an important promoter of tumour growth and metastasis.[1,2] Moreover, activation of IGF-IR may contribute to tumour angiogenesis by up-regulating the expression of vascular endothelial growth factor (VEGF) in certain cancer entities, including pancreatic cancer.[3,4] In addition, we recently demonstrated that IGF-IR may also up-regulate the expression of cyclooxygenase-2 (COX-2) in pancreatic cancer, yet another important mechanism for modulating tumour

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angiogenesis and metastasis.[5] Due to the aggressive course of pancreatic cancer, and continued lack of potent therapeutic strategies, there is a great need for development of novel approaches for improving patient outcome. These novel strategies will likely involve molecular targeted therapy to improve efficacy of existing concepts.

For treatment of pancreatic cancer, interference with the IGF-IR function certainly appears to be one such promising molecular approach, as frequent overexpression of IGF-IR and its ligands (IGF-I, -II) has been demonstrated in human pancreatic cancer.[4,6,7] Moreover, molecular mechanisms have been identified that lead to an autocrine activation of the IGF-I receptor, which in addition could substantially contribute to tumour progression and angiogenesis.[4,8,9] In a previous study, we were able to prove that IGF-IR is indeed a valid target for therapy of this aggressive cancer entity, since blockade of IGF-IR by stable transfection with a dominant-negative plasmid resulted in a significant reduction of orthotopic tumour growth and angiogenesis.[4] However, the development of specific inhibitors to IGF-IR that could be used therapeutically has been challenging, as anti-IGF-IR antibodies either led to an initial activation of IGF-IR,[10] or cross-reacted with the insulin receptor (IR).[2] Nevertheless, successful design of therapeutic inhibitors to IGF-IR has recently been reported in studies showing promising anti-neoplastic efficacy of these agents in some experimental models.[11–14] To date, therapeutic inhibition of IGF-IR has either been achieved by using function-blocking antibodies,[11,14–18] or with small-molecule tyrosine kinase inhibitors.[12,19,20] The latter comprises the IGF-IR inhibitor NVP-AEW541 (Novartis Pharma, Basel, Switzerland), which is capable of discriminating between IGF-IR and the insulin receptor.[12] Anti-neoplastic efficacy of NVP-AEW541 has recently been shown in experimental models of acute myeloid leukaemia,[21] neuroblastoma[20] and Ewing's sarcoma,[13,19] which makes NVP-AEW541 an interesting substance to be used in a clinical setting. However, the growth-inhibitory potential of any IGF-IR blocking substance, including NVP-AEW541, has to date not been investigated in experimental models of human pancreatic cancer.

Based on results from translational studies, clinical trials with IGF-IR inhibitors have already been initiated for treating solid malignancies, such as colon cancer and breast cancer. Since IGF-IR represents a valid target in pancreatic cancer, and given that tumour microenvironment plays a crucial role for efficacy of targeted therapy, we investigated effects of IGF-IR inhibition with NVP-AEW541 in an orthotopic model of pancreatic cancer *in vivo*, with the future aim of using such inhibitor in clinical trials for therapy of pancreatic cancer. Furthermore, we hypothesised that measuring serum levels of IGF-binding protein-3 (IGFBP-3) could potentially serve as a biologic marker for monitoring efficacy of NVP-AEW541 *in vivo*, as IGFBP-3 represents an important modulator of the IGF-I/IGF-IR axis.[22–25]

2. Materials and methods

2.1. Cell culture and reagents

The human pancreatic cancer cell lines HPAF-II and BxPC-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and the metastatic L3.6pl cell line was kindly provided by Dr. I.J. Fidler (The University of Texas MD Anderson Cancer Center, Houston, TX). Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Gibco, Karlsruhe, Germany) supplemented with 15% foetal calf serum (FCS) and maintained in 5% CO_2 at 37 °C, as described.[4,26] Endothelial cells were obtained from PromoCell (Heidelberg, Germany), and cultured as recommended by the manufacturer. The IGF-IR inhibitor NVP-AEW541 was provided by Dr. J. Zimmermann (Novartis, Basel, Switzerland). For *in vitro* experiments, a 10 mM stock solution was prepared in DMSO. Animals received oral gavages of freshly prepared NVP-AEW541 in 25 mM L(+)Tartaric Acid, as described.[12,21] Recombinant insulin-like growth factor (IGF-I), epidermal growth factor (EGF) and hepatocyte growth factor (HGF) were purchased from R&D Systems (Wiesbaden, Germany).

2.2. Western blot analysis

Unless otherwise indicated, cells were incubated with NVP-AEW541 (1 μM) 1 h prior to stimulation with IGF-I (100 ng/ml). Whole cell lysates were prepared, as described elsewhere.[4] Protein samples (50 μg) were subjected to Western blotting on a denaturating 10% SDS w/v gel elecrophoresis. Membranes were sequentially probed with antibodies (1:1000) to indicate signalling intermediates, as described.[4] Antibodies to phospho-Akt^{Ser473}, Akt, phospho-Erk$^{Thr202/Tyr204}$, Erk, phospho-STAT3^{Tyr705}, STAT3, and phospho-IGF-IR^{Tyr1131} were purchased from Cell Signaling Technologies (Beverly, MA), and antibodies against β-actin and IGF-IRβ were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-phospho-IRS-1^{Tyr612} was obtained from Calbiochem (Merck, Darmstadt, Germany). Western blot analysis of tumour samples was performed likewise after tissue lysis using extraction buffer. For analysis of IGF-IR expression in tumour specimens, immunoprecipitation of protein samples (800 μg) was performed using an anti-IGF-IRβ antibody (Santa Cruz Biotechnologies).[4]

2.3. Enzyme-linked immunosorbent assays for VEGF and IGFBP-3

To determine changes in VEGF in cell culture supernatants and serum samples from mice, we used an ELISA kit specific to human VEGF-A (BioSource Europe, Nivelles, Belgium), as human tumour cell lines were used in experiments.[4] Pancreatic cancer cells were plated at 40–50% density and incubated with or without NVP-AEW541 (1 μM) for 48 h. Cells were subsequently counted and analysis of culture supernatants was performed according to the manufacturer's protocol. VEGF protein levels are expressed as pg per 1000 viable cells or as pg/ml serum (*in vivo* experiments). IGFBP-3 in serum samples was measured using an ELISA kit from R&D Systems (Wiesbaden, Germany).

2.4. Real-time PCR analysis for VEGF

For real-time PCR (RT-PCR), total RNA was isolated using Trizol Reagent (Invitrogen, Karlsruhe, Germany) and subsequently purified by ethanol precipitation. For each RNA sample, a

1 μg aliquot was reversely transcribed into cDNA using the Superscript II Kit (Qiagen, Hilden, Germany). Primer pairs were as follows: VEGF165 (5′-GCACCCATGGCAGAAGGAGGAG; 3′-AGCCCCCGCATCGCATCAG), and β-actin (5′-AGAGGGAAATCGTGCGTGAC; 3′-CAATAGTGATGACCTGGCCGT). Primers were optimised for $MgCl_2$ and annealing, and PCR products were confirmed by gel electrophoresis. RT-PCR was performed using the LightCycler system and Roche Fast-Start Light Cycler-Master Hybridisation Probes master mix (Roche Diagnostics, Mannheim, Germany).

2.5. MTT assay

To evaluate cytotoxic effects of NVP-AEW541 on cancer cells and endothelial cells, either L3.6pl, HPAF-II cells or HUVECs were seeded into 96-well plates (1×10^3/well; 12 wells per condition) and exposed to various concentrations of NVP-AEW541 for specified times, respectively. We used the methylthiazole tetrazolium (MTT) assay to assess cell viability, as previously described.[4] Experiments were performed in triplicate. Changes in O.D. are expressed in % as relation to a determined base-line O.D. (overnight incubation of cells).

2.6. Cell death detection ELISA assay

The cell death detection ELISA^{Plus} kit (CDD, Roche, Germany) was used to measure DNA fragmentation as a marker for apoptosis. Cells were seeded into 96-well plates at 10,000 cells per well. After 24 h, cells were treated with NVP-AEW541 (1 μM) and grown for an additional 24 and 48 h in RPMI1640 containing 20% FCS w/w. Cytoplasmatic fractions were prepared using the provided kit (lysis buffer) and protein of control and treated cells were transferred into streptavidin-coated 96-well plates and incubated with biotinylated mouse anti-histone antibody and peroxidase-conjugated mouse anti-DNA antibody at room temperature for 2 h, according to the manufacturer's protocol. Absorbance was determined at 405 nm to 490 nm. To calculate the specific enrichment of mono- and oligonucleosomes released into the cytoplasm (enrichment factor) of the treated cells, the following formula was used: mU of the sample (dying/dead cells)/ mU of corresponding negative control.[27]

2.7. Migration assays

To determine the impact of NVP-AEW541 treatment on cancer cell motility *in vitro*, migration assays were performed using modified Boyden chambers, as described.[28] Briefly, 5×10^4 cells were resuspended in 1% FCS-DMEM w/w and seeded into inserts with 8 μm filter pores (Becton Dickinson Bioscience, Heidelberg, Germany). As chemoattractant, 10% FCS w/w ± IGF-I (100 ng/ml) was used. After 48 h, cells were fixed and migrated cells stained (Diff-Quick reagent, Dade Behring, Newark, NJ) and counted in four random fields.

2.8. Animal models

Eight-week-old male athymic nude mice (BALB/$c^{nu/nu}$) (Charles River, Germany) were used for experiments, as approved by the Institutional Animal Care and Use Committee of the University of Regensburg and the regional authorities. In addition, experiments were conducted according to 'Guidelines for the Welfare of Animals in Experimental Neoplasia' published by The United Kingdom Coordinating Committee on Cancer Research. The effects of IGF-IR inhibition on the growth of human pancreatic cancer cells (BxPC-3) were first investigated in a subcutaneous xenograft tumour model. Cancer cells (1×10^6) were injected into the subcutis (right flank) of nude mice. Mice were randomised and assigned to treatment groups ($n = 3$–5/group). Treatment with NVP-AEW541 (50 mg/kg, bid, p.o.) was initiated on day 5 when tumours became palpable. Tumour diameters were measured every other day and tumour volumes calculated (width$^2 \times$ length $\times$ 0.5). When the experiment was terminated, blood was taken from anaesthetised mice. Mice were then sacrificed and subcutaneous tumours excised, weighed, and prepared for Western blot analysis. Blood glucose levels were measured using a digital glucose reader (Department of Surgery).

Effects of NVP-AEW541 were subsequently evaluated in an orthotopic pancreatic cancer model using male athymic nude mice (BALB/$c^{nu/nu}$) (Charles River), as described previously.[4] In brief, 1×10^6 human pancreatic cancer cells (L3.6pl) were injected into the pancreas of mice. After implantation, tumours were allowed to grow 7 days before treatment was initiated. Mice were randomised into groups receiving either vehicle or NVP-AEW541 (50 mg/kg, bid, p.o.). On day 21 after tumour cell inoculation, mice were anaesthetised, serum was collected, and animals were then sacrificed. Excised tumours were measured and weighed, and tissues snap frozen for PCR analysis. For immunohistochemical analysis, tumours were embedded in optimum cutting temperature (OCT) medium.

2.9. Immunohistochemical analysis of tumour vascularisation

Multiple cryo-sections were obtained from tumours for all immunohistochemical analysis. CD31-positive vessel area was assessed using rat anti-mouse CD31/PECAM-1 antibody (Pharmingen, San Diego, CA) and peroxidase-conjugated goat anti-rat IgG (Jackson Research Laboratories, West Grove, PA), as previously described.[4] Images were obtained in four different quadrants of each tumour section (2 mm inside the tumour–normal tissue interface) at 40× magnification. Measurement of vessel area of CD31-stained vessels was performed by converting images to greyscale and setting a consistent threshold for all slides using ImageJ software (version 1.33; National Institute of Health, Bethesda, MD). Vessel areas were expressed as pixels per high-power field (HPF).[28]

2.10. Statistical analysis

Statistical analysis was performed using SigmaStat (Version 3.0). Results of *in vivo* experiments were analysed for significant outliers using the Grubb's test for detecting outliers (www.graphpad.com). Tumour-associated variables (weight, tumour volume, CD31-area) in *in vivo* experiments were tested for statistical significance using the Mann–Whitney U test for non-parametric data. The two-sided student's *t*-test was applied for analysis of *in vitro* data. All results are

expressed as the mean ± SEM, unless stated otherwise in the legends.

3. Results

3.1. *Effect of NVP-AEW541 on IGF-I-mediated signalling in human pancreatic cancer cells*

We first investigated whether NVP-AEW541 could interfere with the activation of signalling pathways in human pancreatic cancer cells, which are known to be involved in the IGF-I/IGF-IR signalling cascade. Results from *in vitro* experiments were validated in three different cell lines; however, results from HPAF-II cells are illustrated throughout the manuscript. For this purpose, cells were incubated (24 h) with various doses of NVP-AEW541 and subsequently stimulated with IGF-I (10 min). Western blot analysis shows that stimulation with IGF-I leads to activation of IGF-IR, IRS-1, MEK/Erk and Akt (Fig. 1A). Blocking IGF-IR with NVP-AEW541 resulted in a dose-dependent inhibition of IGF-IR and IRS-1 substrate, and in a marked reduction of phosphorylation of down-stream signalling pathways (Fig. 1A). However, to reduce the possibility of unspecific signalling pathway inhibition by the small molecule inhibitor, an *in vitro* dose of 1 μM NVP-AEW541 was selected for subsequent assays. Moreover, to validate these IGF-IR-inhibitory effects, we reduced the pre-treatment time for NVP-AEW541 to 1 h. Similarly, results demonstrate that effective inhibition of IGF-IR and associated down-stream signalling pathways can be achieved by short-term treatment with NVP-AEW541 (Fig. 1B). Interestingly, NVP-AEW541 also harbours the potential to diminish constitutive and inducible phosphorylation of the transcription factor STAT3 in pancreatic cancer cells (Fig. 1B,C). Furthermore, a robust inhibition of IGF-IR and signalling intermediates, including the STAT3 pathway, was also seen when cells were pre-treated with NVP-AEW541 (1 h; 1 μM) and subsequently stimulated for various times with IGF-I (Fig. 1C). Since the IGF-IR system has been shown to be important for regulating the VEGF expression in pancreatic cancer cells,[4] the impact of NVP-AEW541 on VEGF secretion was measured by ELISA. Results from these analyses show that treatment of pancreatic cancer cells with NVP-AEW541 leads to a substantial

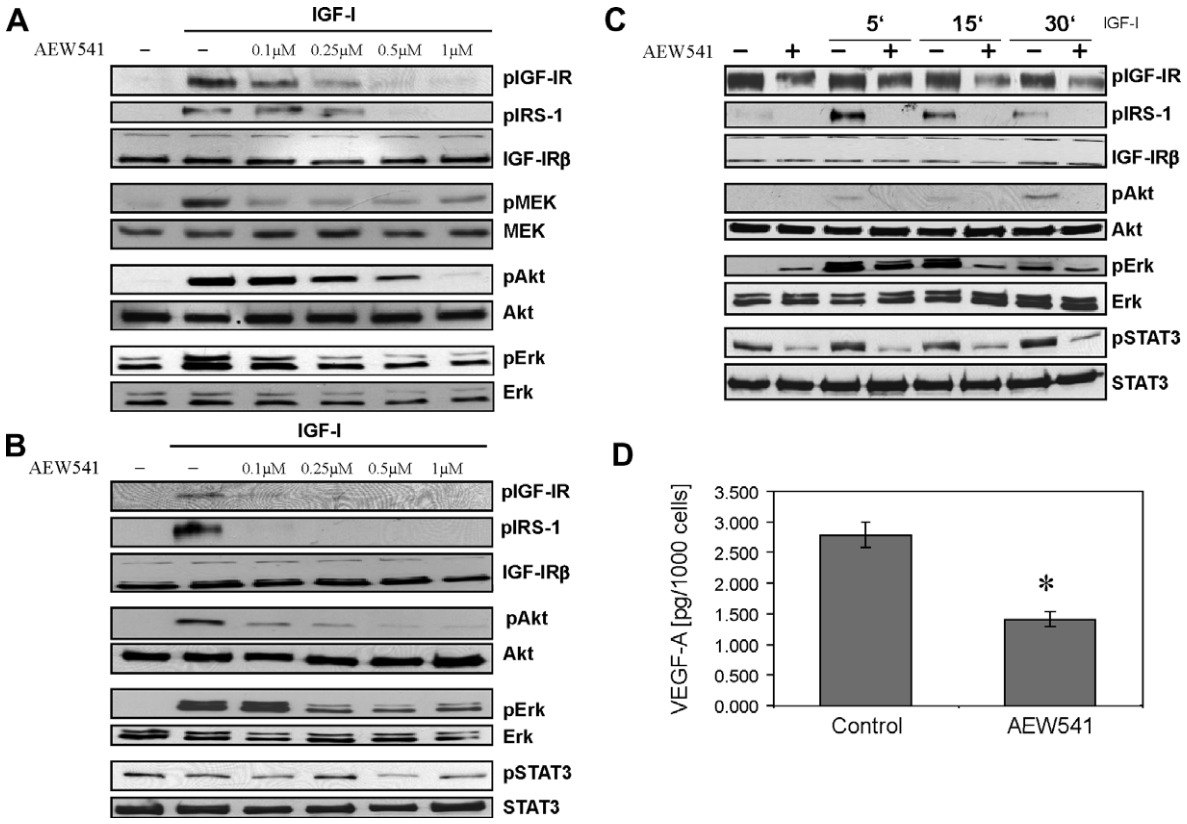

Fig. 1 – Effect of NVP-AEW541 on signalling pathway activation in pancreatic cancer cells. HPAF-II cells were treated with NVP-AEW541 and subsequently stimulated with IGF-I to determine the impact on cell signalling by Western blotting. A) HPAF-II cells were incubated with indicated doses of NVP-AEW541 for 24 h prior to stimulation with IGF-I (100 ng/ml). NVP-AEW541 led to a dose-dependent inhibition of the IGF-IR associated pathways IRS-1, MEK, Erk and Akt. B) Similarly, pre-treating HPAF-II cells for 1 h with NVP-AEW541 sufficiently blocked IGF-IR down-stream signalling pathways in a dose-dependent manner. NVP-AEW541 also led to a reduction in STAT3 activation in tumour cells. C) HPAF-II cells were pre-treated for 1 h with NVP-AEW541 (1 μM) and subsequently stimulated with IGF-I for the indicated time. Blocking IGF-IR with NVP-AEW541 substantially reduced signalling pathway activation, including STAT3. D) HPAF-II cells were treated for 48 h with NVP-AEW541 (1 μM) and supernatants analysed for VEGF by ELISA. NVP-AEW541 led to a significant reduction in VEGF secretion (n = 3/group), as calculated in pg protein per 1000 viable cells (*$P < 0.05$). Bars: SEM.

reduction in VEGF secretion (Fig. 1D). This decrease in VEGF was also confirmed on an mRNA level, as determined by real-time PCR (data not shown). We conclude from these experiments that NVP-AEW541 is suitable for inhibition of IGF-IR in pancreatic cancer cells, which in turn leads to a reduction in VEGF expression, suggesting that NVP-AEW541 has potential to reduce human pancreatic cancer growth and angiogenesis.

3.2. *Impact of IGF-IR inhibition on proliferation and migration of pancreatic cancer cells*

The potential cytotoxic effects of NVP-AEW541 on pancreatic cancer cells were determined by MTT analysis. Full media (15% FCS) was used to investigate the impact of NVP-AEW541 on tumour cell proliferation, and serum-reduced conditions (1% FCS) were chosen to evaluate the effects on cell survival *in vitro*. Results showed that NVP-AEW541 markedly reduces tumour cell numbers in a dose-dependent manner after incubation for 48 or 72 h in either 15% FCS or 1% FCS, suggesting that both cell proliferation and survival can be impaired by blocking IGF-IR (Fig. 2A, B). The significant anti-proliferative effect of NVP-AEW541 was also seen when IGF-I was present in culture media (data not shown). However, substantial pro-apoptotic effects of NVP-AEW541 on cancer cells (HPAF-II, L3.6pl) were not detectable in a cell death detection ELISA (CDD) when cells were incubated in full serum medium for 48 h (data not shown). Regarding cell motility, NVP-AEW541 led to a significant inhibition of basal and IGF-I-mediated pancreatic cancer cell migration (Fig. 2C). Importantly, in another assay using HGF (scatter factor) as a chemoattractant, NVP-AEW541 did not alter HGF/cMet-mediated cancer cell migration, hence providing evidence for specificity to the IGF-IR pathway and for the viability of migrated NVP-AEW541-treated cells (data not shown). Together these results suggest that NVP-AEW541 not only decreases pancreatic cancer cell growth, but also harbours the potential to impair pancreatic cancer metastasis.

3.3. *Effect of NVP-AEW541 on endothelial cells*

We next investigated whether NVP-AEW541 could also elicit antiangiogenic effects through direct interaction with endothelial cells (ECs). To test this hypothesis, we used migration assays and MTT analysis to determine the functional impact of NVP-AEW541 on HUVECs. Since EGF also plays an important role in endothelial cell migration and proliferation,[29] we used both EGF and IGF-I to stimulate HUVEC *in vitro*. Moreover, studies have suggested a functional interplay of EGFR with IGF-IR, hence offering the possibility that blocking IGF-IR with NVP-AEW541 could also affect EGFR function.[30] Results show that EGF and IGF-I both lead to a marked increase in endothelial cell migration after stimulating for 4 h (Fig. 3A). Importantly, NVP-AEW541 (4 h, 1 μM) not only decreases basal migratory activity of HUVEC cells, it also significantly impairs EGF- and IGF-I-mediated EC migration *in vitro* (Fig. 3A). In MTT analysis, treating ECs with NVP-AEW541 (1 μM) led to a decrease in cell proliferation regardless of the presence or absence of either EGF or IGF-I. However, despite reaching statistical significance, blocking IGF-IR does not seem to have a major effect on proliferation or survival of endothelial cells (Fig. 3B). We conclude from these experiments that NVP-AEW541 does elicit relevant

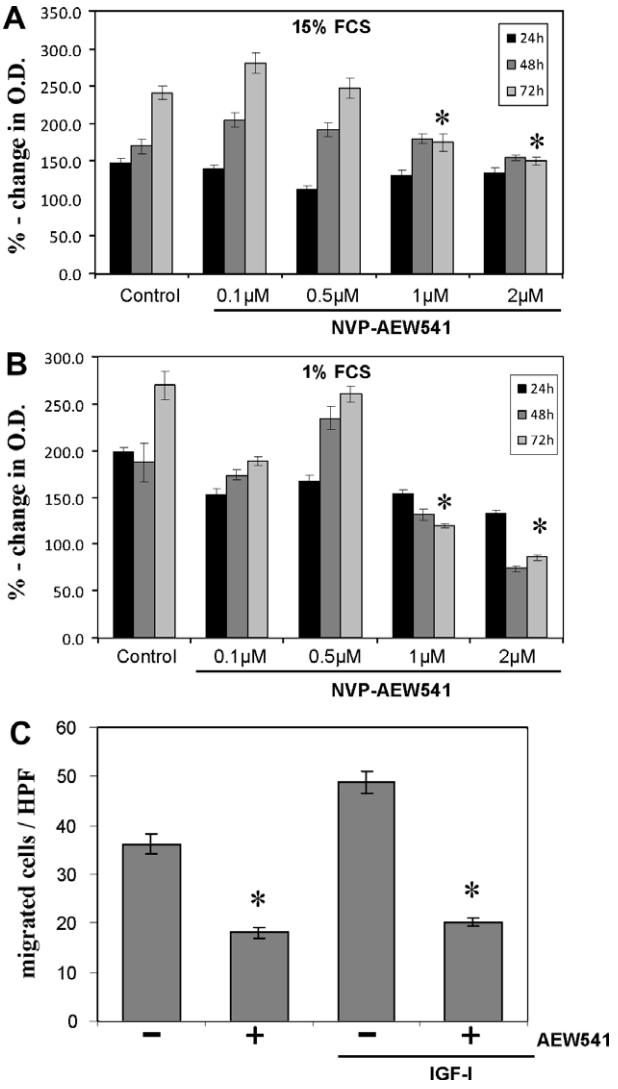

Fig. 2 – Impact of NVP-AEW541 on cell proliferation and migration *in vitro*. MTT analysis was performed to assess the effect of NVP-AEW541 on cell proliferation and survival. Results are expressed in percentage of change in O.D. compared to an initial base line. A) HPAF-II cells were incubated for the indicated time and doses of NVP-AEW541 in 15% FCS. NVP-AEW541 at 1–2 μM significantly reduced cell numbers after 72 h, compared to control (*$P < 0.01$). B) Similarly, HPAF-II cells were incubated for the indicated time and doses of NVP-AEW541 in serum-reduced (1% FCS) conditions. NVP-AEW541 at 1–2 μM significantly reduced cell numbers after 72 h, compared to control (*$P < 0.01$). C) Effect of NVP-AEW541 on cancer cell migration. HPAF-II cells were seeded into modified Boyden chambers with 15% FCS ± IGF-I (100 ng/ml) as a chemoattractant. NVP-AEW541 (1 μM, 48 hours) significantly reduced the migration of pancreatic cancer cells and blunted the pro-migratory effect of IGF-I (*$P < 0.01$). Bars: SEM.

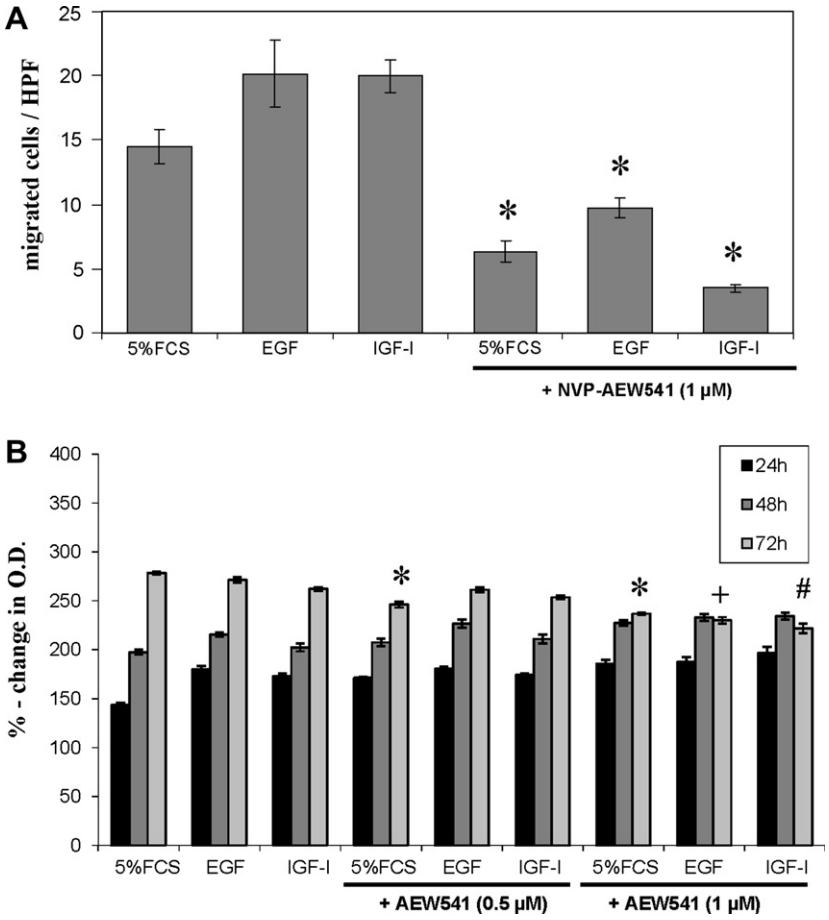

Fig. 3 – Effect of NVP-AEW541 on endothelial cells. The impact of IGF-IR inhibition on endothelial cells (HUVEC) was evaluated in migration assays and by MTT analysis. A) Changes in cell migration by treatment with NVP-AEW541 (1 μM, 4 h) were determined using modified Boyden chambers and 5% FCS as a chemoattractant. Pro-migratory effects of EGF (40 ng/ml) or IGF-I (100 ng/ml) were additionally investigated. After 4 h, NVP-AEW541 significantly reduced EC migration and markedly suppressed pro-migratory properties of both EGF and IGF-I, compared to controls (*$P < 0.01$). B) By MTT analysis, NVP-AEW541 (1 μM) significantly reduced cell proliferation, regardless of presence or absence of either EGF (40 ng/ml), or IGF-I (100 ng/ml) in the culture medium (5% FCS) (*$P < 0.01$ versus 5%FCS; $^{+}P < 0.01$ versus EGF; $^{\#}P < 0.01$ versus IGF-I; for all at the 72 h time point). Bars: SEM.

biologically effects on endothelial cells by impairing EC migration, which could substantially impact tumour angiogenesis in pancreatic cancer.

3.4. *Effects of IGF-IR inhibition on pancreatic cancer growth and vascularisation in vivo*

The effect of IGF-IR inhibition using NVP-AEW541 (50 mg/kg; bid, p.o.) on the growth of BxPC-3 pancreatic cancer cells *in vivo* was first determined in a subcutaneous xenograft model. Results show that blocking IGF-IR significantly reduces tumour growth rates in this model (Fig. 4A). The growth-inhibitory effect of NVP-AEW541 is also reflected by final weights of excised tumours on day 15 (Fig. 4B). Interestingly, treatment (10 days) with NVP-AEW541 resulted in slightly elevated blood glucose levels in treated mice without impacting mouse body weights, which statistically did not differ among groups (Fig. 4C). Moreover, the anti-IGF-IR effect of NVP-AEW541 was also validated by Western blot analysis of tumour specimens, showing that phosphorylation of IGF-IR is substantially diminished (Fig. 4D). To test whether IGF-IR inhibition with NVP-AEW541 indeed reduces pancreatic cancer growth in the appropriate tumour microenvironment *in vivo*, we used an orthotopic model of pancreatic cancer (L3.6pl cells).[4] Mice received either NVP-AEW541 (50 mg/kg; bid, p.o.), or vehicle, starting on day 7 post tumour cell implantation. On day 21 the experiment was terminated as mice in the control group became moribund because of tumour burden. Analysis of pancreatic tumour burden (tumour weight) shows that mice in the NVP-AEW541 therapy arm had developed significantly smaller tumours (60% reduction), as compared to mice in the control group (Fig. 5A). However, mice in any group did not develop distant metastases (liver, lymph nodes), which is similar to our previous model.[4] Importantly, the potent growth inhibitory effect of NVP-AEW541 was accompanied by a moderate decrease in mouse body weight (16%), as compared to mice in the control arm; however, animals did not appear to be compromised by this weight loss. Furthermore, similar to our previous finding that blocking IGF-IR leads to reduced tumour angiogenesis[4],

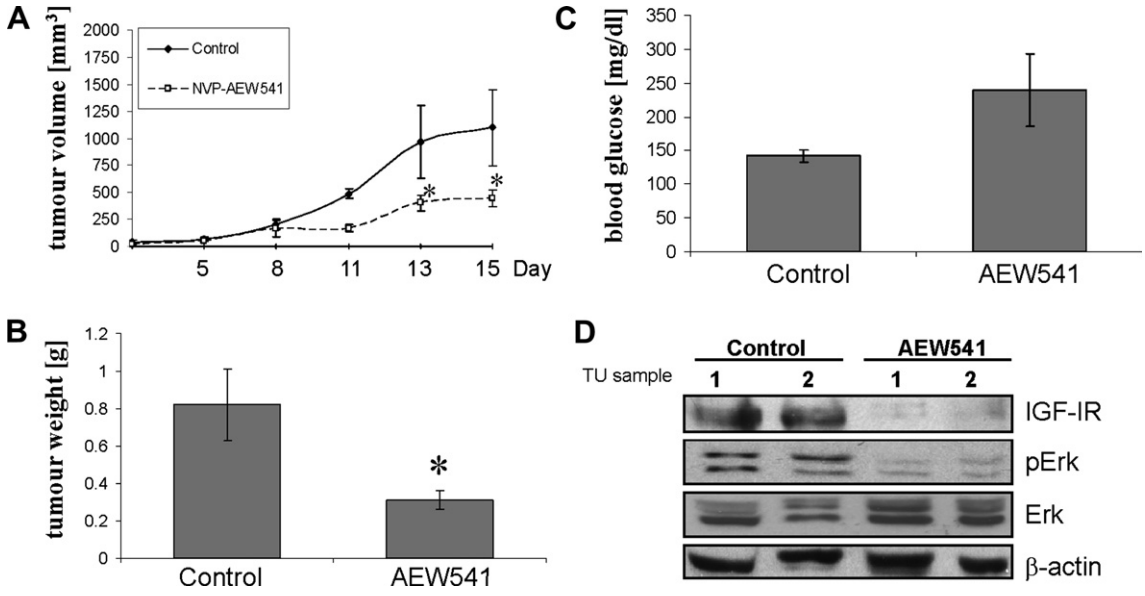

Fig. 4 – Impact of NVP-AEW541 on subcutaneous pancreatic tumour growth. Efficacy of NVP-AEW541 was first determined in a subcutaneous tumour model using BxPC-3 pancreatic cancer cells, where mice received either NVP-AEW541 (50 mg/kg, bid) or vehicle on day 5 after tumour cell inoculation (n = 3–5/group). A) Treatment with NVP-AEW541 significantly reduced tumour growth rates *in vivo* (*$P < 0.05$). B) Final tumour weights (day 15) of excised tumours were significantly lower in the NVP-AEW541 group (*$P < 0.05$). C) Analysis of blood glucose levels in mice showed that there was a trend towards elevated blood glucose levels after 10 days of therapy in mice in the NVP-AEW541 treatment arm (not significant). D) Immunoprecipitation and Western blot analysis of tumour tissues showed that treatment with NVP-AEW541 markedly diminished the phosphorylation of IGF-IR and Erk in tumours. Representative results of two tumours per group are shown.

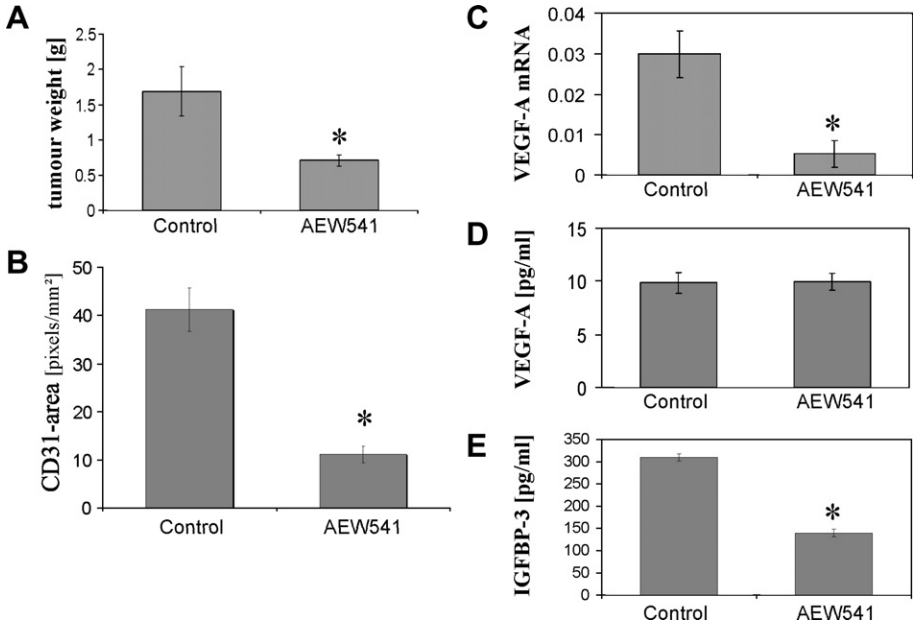

Fig. 5 – Effects of NVP-AEW541 on orthotopic pancreatic tumour growth and vascularisation *in vivo*. The effects of NVP-AEW541 on tumour growth were investigated in the orthotopic L3.6pl tumour model in mice (n = 5/group), where animals were treated with either NVP-AEW541 (50 mg/kg; bid; p.o.) or vehicle 7 days after tumour cell implantation. A) The experiment was terminated on day 21 and weights of excised pancreatic tumours showed that NVP-AEW541 significantly reduced orthotopic tumour growth (*$P < 0.01$). B) Blocking IGF-IR with NVP-AEW541 led to a substantial reduction in vascularisation (CD31-positive vessel area) of pancreatic tumours (*$P < 0.01$). C) NVP-AEW541 treated tumours showed a reduced VEGF mRNA expression, compared to tumours of the control group (*$P < 0.05$). D) In contrast, treatment with NVP-AEW541 did not alter serum VEGF levels. E) Inhibition of IGF-IR with NVP-AEW541 led to significantly reduced serum IGFBP-3 levels in mice, compared to controls (*$P < 0.01$). Bars: SEM.

NVP-AEW541-treated tumours demonstrated a marked reduction in CD31-positive vascular area versus controls (Fig. 5B). This difference in vascularisation was also reflected by RT-PCR results from tumours, demonstrating that treatment with NVP-AEW541 dramatically reduces VEGF mRNA expression in pancreatic tumours (Fig. 5C). In contrast, serum levels of VEGF, as determined by VEGF-ELISA (human VEGF-A) were not affected by this therapy, despite a significantly reduced tumour burden (Fig. 5D). Nevertheless, appropriate biologic markers are required for monitoring IGF-IR targeted therapy. Since IGF-binding proteins, such as IGFBP-3, are important modulators of IGF-I activity, we hypothesised that NVP-AEW541 could have an impact on IGFBP-3 levels in the serum, as IGFBP-3 might reflect biologic changes in pancreatic cancer cells (IGF-IR function).[23–25] An analysis of mouse serum showed that IGFBP-3 levels were significantly reduced in the NVP-AEW541 treatment group (Fig. 5E). Together, we conclude that therapy with NVP-AEW541 harbours the potential to effectively inhibit the growth and vascularisation of human pancreatic cancer, and propose that measuring IGFBP-3 could be a valuable biomarker for efficacy of anti-IGF-IR targeted therapy in pancreatic cancer.

4. Discussion

In this study we demonstrate that the IGF-IR inhibitor NVP-AEW541 effectively blocks IGF-IR function and IGF-I-mediated proangiogenic signalling cascades in pancreatic cancer cells *in vitro*, which translates into a potent inhibition of tumour growth and vascularisation in an orthotopic pancreatic cancer model *in vivo*. Importantly, the additional antiangiogenic properties of NVP-AEW541 appear not to be solely mediated through reducing VEGF expression in cancer cells, but also through direct effects on endothelial cells in terms of impairing cell migration. Moreover, we identified IGFBP-3 serum levels to be differentially regulated by NVP-AEW541, suggesting that measuring IGFBP-3 could potentially be used for monitoring efficacy of NVP-AEW541 in clinical trials.

We and others have demonstrated that, in pancreatic cancer, the IGF-IR system represents an attractive target for cancer therapy.[4,6,7] However, to date, growth-inhibitory effects of any of the IGF-IR-inhibiting substances have not been investigated in preclinical models of pancreatic cancer. We therefore addressed this issue by using NVP-AEW541, a potent IGF-IR tyrosine kinase inhibitor, which has shown promising efficacy in various experimental tumour models, including fibrosarcoma,[12] neuroblastoma,[20] and Ewing's sarcoma.[13] The significant growth inhibitory effects of NVP-AEW541 were either achieved by monotherapy with AEW541 alone,[12,20] or by combining NVP-AEW541 with selected chemotherapeutic agents, such as vincristine in musculoskeletal tumour models.[19] Importantly, NVP-AEW541 has been demonstrated to be highly selective for the IGF-IR system, without significant cross-reaction with the insulin receptor (IR).[12] However, Manara and colleagues very recently demonstrated that NVP-AEW541 not only inhibits migration, metastasis, vascularisation, and growth of Ewing's sarcoma in an experimental model, but also that NVP-AEW541 treatment decreases blood glucose serum levels.[13] Moreover, the authors detected an initial weight loss in mice with NVP-AEW541 treatment, which was re-compensated over time and was comparable to the vincristine chemotherapy arm. In contrast to their study, we observed that NVP-AEW541 (50 mg/kg; bid) led to a modest increase in blood glucose levels (subcutaneous tumour model), and to a moderate loss (16%) in mouse body weight (orthotopic tumour model). These findings could suggest that the insulin/IR axis could indeed be affected by treatment with NVP-AEW541. Nevertheless, we propose that this unspecific effect could in part be responsible for the potent growth inhibition achieved by this IGF-IR inhibitor, as the IR system harbours the potential to partially 'compensate' the loss in IGF-IR function, hence a modest cross-reaction of NVP-AEW541 with the IR might even be beneficial for cancer therapy. Moreover, we consider this potential side-effect of NVP-AEW541 on the IR system to be clinically manageable in patients and a substantial weight loss is not expected. Results from clinical studies using other IGF-IR inhibitors will probably provide some answers to this question, as pre-clinical models will not be sufficient to predict the real impact of IGF-IR targeted therapy on glucose metabolism in patients.[31]

Interestingly, we also found that blocking IGF-IR using NVP-AEW541 decreases STAT3 phosphorylation in pancreatic cancer cells, a finding that has not been previously reported. The transcription factor STAT3 is known to be one of the key regulators of pancreatic tumour growth and angiogenesis, and thus represents an interesting molecular target itself.[32] However, we speculate that the effect on STAT3 is mediated through inhibition of up-stream signalling components and probably in part through a reduction in HIF-1 activity (component of the IGF-I signalling axis), as suggested by our previous studies.[4,5] Importantly, we also discovered a direct effect of NVP-AEW541 on endothelial cells in terms of diminishing VEGF-mediated endothelial cell migration. Together with the detected down-regulation in VEGF expression in tumour cells and pancreatic tumour tissues, these results suggest that NVP-AEW541 harbours antiangiogenic properties in addition to mediating cell cycle arrest and pro-apoptotic effects, as reported by other groups.[33]

Since identification of appropriate biomarkers for monitoring efficacy of IGF-IR targeted therapy remains a major challenge, we tested whether blocking IGF-IR function with NVP-AEW541 could potentially alter serum levels of IGFBP-3, which is an important modulator of the IGF-I biologic activity. In further support of this idea, elevated serum IGFBP-3 has been associated with an increased risk of pancreatic cancer death.[23,24] Moreover, when Lin and colleagues analysed serum from patients ($n = 69$) who died from pancreatic cancer, they found levels of IGF-I to be positively correlated with IGFBP-3. The authors determined that the risk of pancreatic cancer death increased significantly with increasing serum levels of IGFBP-3, and thus concluded that high serum levels of IGF-I and IGFBP-3 could be associated with an increased risk of death from pancreatic cancer.[24] In another study, Karna and colleagues investigated pancreatic cancer tissues and serum of patients with pancreatic cancer for IGF-I and IGFBPs. This group showed that in pancreatic cancer, IGF-I, IGFBP-3, and IGFBP-1 levels in serum were significantly increased, accompanied by an up-regulation of IGF-IR expression in tumour tissues, suggesting that elevated serum levels of

IGF-I, IGFBP-3 and IGFBP-1 may serve as markers of pancreatic cancer.[23] Consistent with these arguments, we found serum IGFBP-3 to be substantially reduced with NVP-AEW541 treatment. We speculate that this reduction either occurs as a consequence of reduction of IGF-IR activity (i.e. inhibition of autocrine signalling), or due to reduced tumour burden. Interestingly, we did not detect such modulation of IGFBP-3 protein in a second pancreatic tumour model, where similar growth inhibition was achieved using a non-IGF-IR targeting molecular compound (target was not IGF-IR), suggesting that alterations of IGFBP-3 by NVP-AEW541 could be a 'specific' phenomenon of targeting the IGF-IR. Moreover, in *in vitro* assays, we detected IGFBP-3 secretion by pancreatic cancer cells, but this was not substantially modulated by NVP-AEW541 treatment (48 h) (data not shown), suggesting that observed changes of IGFBP-3 in serum of mice is more complexly regulated and modulated (i.e. tumour microenvironment). This novel finding needs to be validated and explored further in clinical trials using anti-IGF-IR compounds.

In conclusion, targeting IGF-IR with the tyrosine kinase inhibitor NVP-AEW541 leads to effective growth inhibition in a preclinical model of pancreatic cancer. Together with its additional antiangiogenic properties in terms of reducing VEGF expression and EC migration, inhibitors of IGF-IR might be a valuable addition to anti-neoplastic chemotherapy regimens or to multimodality therapy of pancreatic cancer. The role of measuring serum IGFBP-3 for monitoring efficacy of IGF-IR targeted therapy remains to be validated in clinical trials.

Conflict of interest statement

None declared.

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