The insulin-like growth factor receptor 1 is a promising target for novel treatment approaches in neuroendocrine gastrointestinal tumours

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Abstract

Gastrointestinal neuroendocrine tumours (NET) represent a heterogeneous tumour entity. The anti-neoplastic therapy of advanced NET disease is still unsatisfactory and innovative therapeutic approaches are needed. As NET frequently express insulin-like growth factors (IGFs) and their receptors (IGFR), known to promote survival, oncogenic transformation, tumour growth and spreading, the inhibition of the IGF/IGF-receptor system may offer possibilities for novel targeted treatment strategies of NET. Here, we studied the anti-neoplastic effects of an inhibition of the IGF-I receptor (IGF-1R) signalling in NET cells by the novel IGF-1R tyrosine kinase (TK) inhibitor NVP-AEW541, whose anti-neoplastic potency has not yet been tested in NET disease. Using two human NET cell lines with different growth characteristics, we demonstrated that NVP-AEW541 dose-dependently inhibited the proliferation of NET cells by inducing apoptosis and cell cycle arrest. Anti-neoplastic effects of NVP-AEW541 were also detected in primary cultures of human neuroendocrine gastrointestinal tumours. Apoptosis was characterized by activation of the apoptotic key enzyme, caspase-3, as well as by detection of changes in the expression of the pro- and anti-apoptotic proteins, BAX and Bcl-2, after NVP-AEW541 treatment. Cell cycle was arrested at the G1/S checkpoint. The anti-neoplastic effects of NVP-AEW541 involved the inactivation of ERK1/2. Induction of immediate cytotoxicity did not account for the anti-neoplastic effects of NVP-AEW541, as shown by measurement of lactate dehydrogenase release. Moreover, additive anti-neoplastic effects were observed when NVP-AEW541 was combined with cytostatics such as doxorubicin or the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, fluvastatin. This is the first report on the induction of apoptosis and cell cycle arrest by the IGF-1R-TK inhibitor, NVP-AEW541, in NET cells. The inhibition of the IGF/IGFR system appears to be a promising novel approach for future treatment strategies of NET disease.

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Introduction

Gastrointestinal neuroendocrine (NE) tumours represent a rare and rather heterogeneous tumour entity. Metastatic NE gastrointestinal tumours often release excessive amounts of biogenic amines and/or neuropeptides thereby causing a characteristic hypersecretion

syndrome. The often bizarre clinical symptoms are generally well controlled by somatostatin analogues or interferon- α (IFN α) (Öberg 2001, Scherübl *et al.* 2003). However, growth and spread of neuroendocrine cancers is not well controlled by either biotherapy or chemotherapy. Thus, therapeutic options to inhibit the growth and spread of neuroendocrine gastrointestinal

tumours (NET) are still unsatisfactory. In the light of the particular biology of NET, innovative treatment strategies should be developed that are both effective and well tolerated.

Recently, evidence has been accumulated that both insulin-like growth factors (IGF-I and IGF-II) and their receptor, IGF-1R, are involved in the development and progression of cancer (Baserga 2000, Wang & Sun 2002, Wang *et al.* 2003). The interaction of IGF-I and IGF-II with IGF-1R plays a pivotal role in tumorigenesis, proliferation and spread of many cancers by controlling cell cycle progression, preventing apoptosis as well as by regulating and maintaining the tumorigenic phenotype. Thus, not surprisingly, a wide variety of tumours including gastrointestinal NETs (Wulbrand *et al.* 2000, Zhang & Yee 2004) show abnormal high or constitutive expression of IGFs and IGF receptors (IGFRs). Expression of IGFs and their cognate receptors has been correlated with disease stage, reduced survival, development of metastases and tumour de-differentiation of several tumours (Scharf & Bräulke 2003, Yao *et al.* 2003, Fottner *et al.* 2004) and contributes to the excessive secretion of biogenic amines of gastrointestinal NET (von Wichert *et al.* 2000).

Several approaches have demonstrated the therapeutic potential of interfering with IGF-1R-mediated signalling *in vitro* and *in vivo*. These approaches included the use of IGF-1R blocking antibodies (Scotlandi *et al.* 1998), IGF-1R antisense oligonucleotides (Shapiro *et al.* 1994) or IGF-1R siRNA (Salisbury & Macaulay 2003).

Furthermore, epidemiological evidence for a role of IGF-1R signalling in cancer has emerged from various studies demonstrating increased expression levels of multiple components of the IGF signalling system in diverse tumour types (for reviews see Khandwala *et al.* 2000, Grimberg & Cohen 2000, Yu & Rohan 2000, Fürstenberger & Senn 2002).

Recently, several potent and selective inhibitors of IGF-1R tyrosine kinase (IGF-1R-TK) have been introduced as promising novel agents for cancer therapy. One of these compounds is the orally available compound NVP-AEW541, a low molecular weight IGF-1R-TK inhibitor belonging to the pyrrolo[2,3-d]pyrimidine class (Scotlandi *et al.* 2005). At the cellular level, NVP-AEW541 was shown to be highly selective for IGF-1R-TK as compared with both the closely related insulin receptor (InsR) and other tyrosine or serine/threonine kinases. Anti-neoplastic properties of both NVP-AEW541 and of specific IGFR antibodies have already been demonstrated in fibrosarcomas, breast cancer and musculoskeletal

tumours (Garcia-Echeverria *et al.* 2004, Scotlandi *et al.* 2005).

IGFR-TK inhibition has not yet been evaluated in the anti-neoplastic treatment of NE tumours. Hence, in the present study, we examined the anti-neoplastic potency of the selective IGF-1R-TK inhibitor, NVP-AEW541, in two distinct NE gastrointestinal tumour cell lines with different growth characteristics. We focused on NVP-AEW541's growth inhibition, induction of apoptosis and cell-cycle arrest in NE gastrointestinal tumour cells. Moreover, we checked for additive anti-neoplastic effects of NVP-AEW541 when combined with conventional cytostatic drugs or the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, fluvastatin. The possible clinical implications of our findings were examined in primary cell cultures of human gastrointestinal neuroendocrine tumours.

Materials and methods

Cell cultures

Human pancreatic carcinoid BON cells, which were established from a human pancreatic carcinoid tumour, provide a useful model to study the biology of neuroendocrine tumours *in vitro* (Evers *et al.* 1994). BON cells were grown in a 1:1 mixture of DMEM and Ham's F-12 medium containing 10% FCS (Biochrom Co., Berlin, Germany) and 1% L-glutamine. The human insulinoma cell line CM (Baroni *et al.* 1999), kindly provided by Professor P Pozzilli (University La Sapienza of Rome, Italy), was cultured in RPMI 1640 supplemented with 5% FCS (Biochrom) and 1% L-glutamine. Both cell lines were kept at 37 °C in a humidified atmosphere (5% CO₂).

Isolated primary cell cultures were established from resection specimens of four histologically verified gastrointestinal NETs of the stomach (*n* = 1), ileum (*n* = 2) or rectum (*n* = 1). The human tumour material was used according to the standards set by the Ethical Committee of the Charité-Universitätsmedizin Berlin. Mechanical dissection was performed with a disaggregation system (Medimachine; Becton Dickinson Co., Heidelberg, Germany) as described elsewhere in detail (Glassmeier *et al.* 1997, Höpfner *et al.* 2003). Cells were maintained in Earle's 199 medium (Biochrom Co.) containing 20% fetal calf serum, 2 mM L-glutamine, 2% (vol/vol) Biotect protective medium (Biochrom Co.), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% vol/vol amphotericin B. Cells were kept in an incubator at 37 °C in a humidified atmosphere of 5% CO₂. Half the medium was changed every day. Cells

remained in culture for at least 2 days before the experiments were carried out. Trypan blue exclusion tests revealed that the proportion of dead cells never exceeded 5%.

Drugs

NVP-AEW541 was a kind gift from Novartis, Basel, Switzerland. Doxorubicin and 5-fluorouracil (5-FU) were purchased from Sigma (St Louis, MO, USA). Fluvastatin was purchased from Calbiochem (Bad Soden, Germany). Stock solutions were prepared in DMSO and stored at -20°C ; they were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration was $<0.1\%$.

Measurement of growth inhibition

Drug-induced changes in cell numbers of BON and CM cells were evaluated by crystal violet staining, as described by Huether *et al.* (2005). In brief, cells in 96-well plates were fixed with 1% glutaraldehyde. Then, cells were stained with 0.1% crystal violet. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 0.2% Triton-X-100. Light extinction, which increases linearly with the cell number, was analysed at 570 nm using an ELISA-Reader.

Determination of cytotoxicity

Cells were seeded at a density of 5000 cells/well into 96-well microtitre plates and incubated with rising concentrations of NVP-AEW541 for 8–24 h. Thereafter, the release of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating cytotoxicity, was determined by using a colourimetric kit from Roche as described elsewhere (Decker & Lohmann-Matthes 1988). Maximum release of LDH was obtained by adding 100 μl 2% Triton X-100 to untreated cells. For determinations, 100 μl of each sample were incubated with 100 μl LDH assay reagent for 10 min at room temperature in the dark. The absorbance of samples was then measured at 490 nm. Percentage of LDH release was determined by dividing released LDH of the cells by maximum LDH release multiplied by 100.

Cell cycle analysis

Cell cycle analysis was performed by the method of Vindelov and Christensen, as described previously (Maaser *et al.* 2001, Höpfner *et al.* 2004a). Cells were trypsinised, washed and the nuclei were isolated using the CycleTest PLUS DNA Reagent Kit (Becton

Dickinson). DNA was stained with propidium iodide according to the manufacturer's instructions. The DNA content of the nuclei was detected by flow cytometry and analysed using CellFit software (Becton Dickinson).

Measurement of caspase-3 activity

Preparation of cell lysates and determination of caspase-3 activity was performed as described (Maaser *et al.* 2002, Sutter *et al.* 2004). The activity of caspase-3 was calculated from the cleavage of the fluorogenic substrate DEVD-AMC (Calbiochem). Cell lysates were incubated with substrate solution (caspase-3 substrate AC-DEVD-AMC 20 $\mu\text{g ml}^{-1}$, HEPES 20 mM, glycerol 10%, dithiothreitol (DTT) 2 mM, pH 7.5) for 1 h at 37°C , and the cleavage of the substrate was measured with a VersaFluor fluorometer (excitation: 360 nm emission: 460 nm) from Biorad (Munich, Germany).

Western blotting

Western blotting was performed as described (Sutter *et al.* 2003, Höpfner *et al.* 2004b). In brief, whole-cell extracts were prepared by lysing cells. Lysates containing 30 μg protein were subjected to gel electrophoresis. Proteins were then transferred to PVDF membranes by electroblotting for 2 h. Blots were blocked in 2.5% BSA solution for 1 h, and then incubated at 4°C overnight with antibodies directed against ERK 1/2 and pERK 1/2 (1:1000 or 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as well as with anti-human Bcl-2 or BAX (1:200 or 1:1000; Novocastra Laboratories, Newcastle-upon-Tyne, UK) and with cyclin D1 and p27 (1:200 or 1:2500; Santa Cruz Biotechnology). For detection of IGF-1R protein expression, a polyclonal antibody (1:1000, Santa Cruz Biotechnology) recognizing the β -chain of IGF-1R (Höpfner *et al.* 2003) was used. The phosphorylated form of IGF-1R was determined by using a polyclonal antibody from Biomol (1:1500; Biomol, Hamburg, Germany). Expression of InsR protein was detected by using a polyclonal antibody from Santa Cruz (1:200; Santa Cruz Biotechnology) recognizing the α -chain of the InsR. HeLa cell lysate (Santa Cruz Biotechnology) was used as positive control for InsR expression. After incubation with horseradish peroxidase-coupled anti-IgG antibodies (1:10000; Amersham, Uppsala, Sweden) at room temperature for at least 1 h, the blot was developed using enhanced chemiluminescent (ECL) detection (Amersham) and subsequently exposed to Hyperfilm ECL film (Amersham) for 0.5–5 min. Experiments

were performed three times, and representative experiments are shown.

Live/Dead assay

Cell viability of NVP-AEW541-treated NET cells was determined using a cell viability/cytotoxicity assay kit (Live/Dead assay) from Molecular Probes (Leiden, The Netherlands) (Höpfner *et al.* 2003). Cells grown on glass coverslips were incubated for 24 h with NVP-AEW541 (0–10 μ M). After washing with PBS, cells were incubated with calcein-AM (320 nM) and ethidium-homodimer-1 (EthD-1) (2 μ M) for at least 1 h at 37 °C. Calcein (ex/em ~495 nm/~510 nm) and EthD-1 (ex/em ~495 nm/~635 nm) fluorescence was used for monitoring living and dead cells with a fluorescence microscope from Zeiss (Axioskop-2; Jena, Germany). Quantification of live and dead cells in each sample was carried out by calculating the average value of at least four arbitrarily chosen image areas of the respective coverslip.

Results

NVP-AEW541-induced dephosphorylation of IGF-1R in gastrointestinal neuroendocrine tumour cells

In a previous study we evaluated the functional expression of IGF-1R in CM insulinoma and BON carcinoid cells (Höpfner *et al.* 2003). Here, we checked for NVP-AEW541-induced dephosphorylation of IGF-1R in neuroendocrine tumour cells. Cells were cultured in serum-free medium for 24 h. Subsequently, cells were stimulated with 100 ng/ml IGF-I for 1 h in the presence of rising concentrations of NVP-AEW541 (0–6 μ M). IGF-1R phosphorylation induced by short-term stimulation with IGF-I was dose-dependently reduced by NVP-AEW541, while the total IGF-1R expression remained unaffected (Fig. 1A). The expression of IGF-I receptors in primary cell cultures established from resected specimens of four human gastrointestinal neuroendocrine tumours was evaluated by immunofluorescence microscopy using a polyclonal anti-IGF-1R antibody (5 μ g/ml, Santa Cruz Biotechnology) (Fig. 1B). Additionally, protein expression of the insulin receptor (InsR) was evaluated. In both cell lines only weak InsR expression was observed as compared with the robust IGF-1R expression (Fig. 1C).

Growth inhibitory effects of NVP-AEW541

Changes in cell number caused by IGF-1R-TK inhibition with NVP-AEW541 were studied by crystal

violet assays. In serum-containing medium, NVP-AEW541 time- and dose-dependently inhibited the growth of both BON and CM cells (Fig. 2A,B). After 96 h of incubation with 0–10 μ M NVP-AEW541 (BON cells) or 0–5 μ M NVP-AEW541 (CM cells), a decrease in cell number of almost 100% was observed. The IC₅₀ values of NVP-AEW541, determined after 48 h, amounted to $3.3 \pm 0.3 \mu$ M and $6.6 \pm 0.6 \mu$ M in CM and BON cells respectively.

In the next step, the anti-proliferative efficacy of NVP-AEW541 on IGF-I-stimulated growth was evaluated. NET cells were serum-starved for 48 h and then stimulated with IGF-I (100 ng/ml) as the only growth factor in the incubation medium. Treatment of IGF-I-stimulated NET cells with NVP-AEW541 also resulted in a dose- and time-dependent growth inhibition (Fig. 2C,D). The respective IC₅₀ values of NVP-AEW541-induced growth inhibition amounted to $1.9 \pm 0.2 \mu$ M in CM cells and $6.4 \pm 0.2 \mu$ M in BON cells as determined after 48 h of continuous incubation.

In line with our findings in permanent cell lines, NVP-AEW541 was also effective in primary culture cells of human neuroendocrine gastrointestinal tumours (stomach, ileum and rectum respectively). After three days of incubation, a significant decrease in cell number as well as morphological changes of primary culture cells were observed by performing Live/Dead assays. The cells appeared shrunken and flat. EthD-1-positive staining revealed that NVP-AEW541 caused a loss of cell membrane integrity indicating cell death or that the cells were in the process of dying (Fig. 2E).

Moreover, we investigated the anti-neoplastic effects of combination treatments of NVP-AEW541 plus cytostatic drugs (doxorubicin, 5-FU), or the HMG-CoA reductase inhibitor, fluvastatin (Fig. 3). When given as a single agent for two days, doxorubicin (10–1000 nM) reduced the growth of CM cells by up to $54 \pm 4\%$ (Fig. 3A), while 5-FU (1–25 μ M) inhibited the growth of gastrointestinal neuroendocrine tumour cells by $39 \pm 3\%$ (Fig. 3C). Combination treatment of CM cells with 3 μ M NVP-AEW541 plus doxorubicin (100–1000 nM) resulted in additive anti-proliferative effects, whereas the combination of NVP-AEW541 plus 5-FU did not lead to an anti-proliferative enhancement. Finally, we evaluated the anti-proliferative efficacy of fluvastatin alone and in combination with NVP-AEW541. Treatment of BON and CM cells with fluvastatin (BON: 1–50 μ M; CM: 0.2–5 μ M) led to a dose-dependent reduction in cell growth of 70% in BON (data not shown) and >90% in CM cells. Combination treatment with NVP-AEW541

Figure 1 NVP-AEW541 induces dephosphorylation of IGF-1R in gastrointestinal neuroendocrine tumour cells. Expression of IGF-1R and NVP-AEW541-induced dephosphorylation of IGF-I-pretreated BON cells (A) was evaluated by western blotting. Cells were grown in serum-free medium for 48 h and subsequently stimulated with IGF-I (100 ng/ml) in the absence or presence of NVP-AEW541. Stimulation with IGF-I results in a considerable increase in phosphorylated IGF-1R, which is decreased by NVP-AEW541 treatment in a dose-dependent manner. The robust expression of IGF-1R protein was neither influenced by NVP-AEW541 treatment nor by the IGF-I stimulation. (B) Immunofluorescence staining of IGF-1R antibody-treated primary neuroendocrine gastrointestinal tumour cells (upper panel). Staining of isotypic control is shown in the lower panel. (C) Expression of InsR protein expression was evaluated by western blotting. Both BON and CM cells displayed only a weak expression of InsR. HeLa cell lysate was used as a positive control.

and fluvastatin led to an additively enhanced growth inhibitory effect (Fig. 3B).

NVP-AEW541 and cell cycle regulation

To test whether induction of cell cycle arrest contributed to the anti-proliferative potency of NVP-AEW541 in gastrointestinal neuroendocrine tumour cells, we performed flow cytometric cell cycle analysis.

NVP-AEW541 arrested both BON and CM cells in the G0/G1 phase of the cell cycle, thereby decreasing the proportion of cells in the S and G2/M phases (Fig. 4).

Proapoptotic effects of NVP-AEW541

To determine the contribution of apoptosis to the observed anti-neoplastic effects of NVP-AEW541, the activation of caspase-3, one of the key enzymes

Figure 3 Anti-proliferative effects of combination treatment with NVP-AEW541 plus doxorubicin, or 5-FU, or fluvastatin. (A) Forty-eight hours incubation with doxorubicin (0–1000 nM) led to a dose-dependent growth inhibition of CM cells by >50%. Combination treatment (48 h) of a sub-IC₅₀ concentration of NVP-AEW541 plus doxorubicin led to slightly over-additive growth inhibitory effects. (B) Seventy-two hours incubation with fluvastatin (0–5 μM) led to a dose-dependent growth inhibition of CM cells by >90%. Combination treatment with sub-IC₅₀ concentrations of fluvastatin and NVP-AEW541 resulted in additive growth inhibition. (C) Forty-eight hours incubation with 5-FU (0–25 μM) dose-dependently reduced the growth of CM cells by up to 40%. However, no additive or enhanced growth inhibitory effect could be observed when NVP-AEW541 was combined with 5-FU. All experiments were performed in serum-containing media. Black bars indicate the values of calculated additive growth inhibition. Data are given as percentage of untreated controls (means ± s.e.m. of 3–4 experiments).

in the apoptotic signalling cascade was evaluated. Incubating BON cells with rising concentrations of NVP-AEW541 (0–7.5 μM) for 48 h induced a significant increase in caspase-3 activity. Comparable results were obtained for CM cells after treatment with

0–5 μM NVP-AEW541 for 24 h (Fig. 5). The apoptotic potential of NVP-AEW541 was confirmed by Live/Dead-fluorescence microscopy which monitored morphological changes due to NVP-AEW541 treatment (Fig. 6). Quantification was obtained by directly

Figure 2 NVP-AEW541 induces growth inhibition. NVP-AEW541 led to a time- and dose-dependent growth inhibition of neuroendocrine gastrointestinal tumour cells, as measured by crystal violet staining. In serum-containing medium the growth of CM cells was inhibited with an IC₅₀ value of $3.3 \pm 0.3 \mu\text{M}$ (A), while BON cells displayed an IC₅₀ value of $6.6 \pm 0.6 \mu\text{M}$ (B) as determined after 2 days of incubation. Data are given as percentage of untreated controls (means ± s.e.m. of at least 4 experiments). In serum-free but IGF-I (100 ng/ml) charged medium, both CM (C) and BON cells (D) showed a marked sensitivity to NVP-AEW541 treatment, which dose-dependently inhibited the growth of both cell lines. The IC₅₀ values amounted to $1.9 \pm 0.2 \mu\text{M}$ in CM cells and to $6.4 \pm 0.2 \mu\text{M}$ in BON cells. Seventy-two hours incubation with NVP-AEW541 reduced the cell number and led to a change in the morphology of primary neuroendocrine tumour cells (E). Viable cells are stained green, while cells with impaired cell membranes appear red. Representative findings out of $n = 4$ investigated primary cultures.

Figure 4 Induction of cell cycle arrest by NVP-AEW541. Incubation of CM (A) and BON (B) cells with rising concentrations of NVP-AEW541 for 24 h (CM) or 48 h (BON) respectively resulted in an accumulation of cells in the G0/G1 phase of the cell cycle. Accordingly, the proportion of cells in the S and G2/M phases decreased. Means of four independent experiments are shown. The difference in the proportion of cells in a particular phase of the cell cycle versus control was significant (* $P < 0.05$) for 1–5 μM NVP-AEW541 (CM cells) and for 1–7.5 μM NVP-AEW541 (BON cells).

counting viable (green) and dead (red) cells in 4 arbitrarily chosen image areas of each sample. Representative data out of $n = 3$ independent experiments are shown. At 1 μM NVP-AEW541 $47 \pm 7\%$ of the cells displayed morphological signs of apoptosis

Figure 5 NVP-AEW541 induces activation of caspase-3. NVP-AEW541-induced activation of caspase-3 was evaluated after 24 h (CM cells) or 48 h (BON cells) of incubation. Upon NVP-AEW541 treatment, BON cells (A) as well as CM cells (B) displayed a dose-dependent increase in caspase-3 activity. Data are given as percentage of untreated control (means $\pm$ S.E.M. of four independent experiments for each cell line).

(apoptotic bodies). At 6 μM NVP-AEW541 DNA fragmentation was recognizable in the staining pattern of EthD-1-positive cells. At 10 μM NVP-AEW541 $96 \pm 3\%$ of the cells displayed an intense red staining, indicating cell death (data not shown).

Cytotoxicity of NVP-AEW541

Cytotoxicity of NVP-AEW541 was determined by measuring LDH release. Incubating gastrointestinal NET cells with NVP-AEW541 (BON: 0–7.5 μM and CM: 0–5 μM) for 8 and for 24 h did not result in any measurable increase in LDH release, indicating that NVP-AEW541 does not directly affect cell membrane integrity and does not have immediate cytotoxic effects even at high concentrations (Fig. 7).

Figure 6 NVP-AEW541 induces cell death of NE gastrointestinal tumour cells. NVP-AEW541-mediated induction of cell death and apoptosis-specific morphological changes of BON cells after 24 h incubation with NVP-AEW541 were determined by Live/Dead-fluorescence microscopy. Viable cells are stained green, while cells with impaired cell membranes appear red. NVP-AEW541 dose-dependently induced an increase in cell death and apoptosis-specific formation of apoptotic bodies as well as DNA fragmentation (white arrows). Phase-contrast images are shown in the upper panel, the corresponding fluorescence micrographs are shown in the lower panel. Representative findings of $n = 3$ independent experiments.

NVP-AEW541 regulates ERK1/2 activity and expression of apoptosis-specific and cell cycle regulating proteins

To explore the signalling pathways modulated by IGF-1R-TK inhibition with NVP-AEW541, we investigated changes in the phosphorylation of ERK1/2, which is a member of the MAP kinase family known to be involved in IGF-1R signalling in non-neuroendocrine tumours (Kurihara *et al.* 2000, Alexia *et al.* 2004). Serum-starved BON cells were pretreated with NVP-AEW541 (0–6 µM) for 1 h and subsequently stimulated for 15 min with 100 ng/ml IGF-I. NVP-AEW541 pretreatment prevented IGF-I-induced ERK1/2 phosphorylation in a dose-dependent manner, whereas the amount of total-ERK1/2 remained unaffected (Fig. 8A). Moreover, we investigated the expression of apoptosis-specific and cell cycle regulating proteins. Using BON cells, we could demonstrate that NVP-AEW541 induced a time-dependent increase in the expression of the pro-apoptotic BAX protein, while simultaneously the expression of anti-apoptotic Bcl-2 decreased (Fig. 8B). Additionally, changes in the expression of the two cell cycle regulating proteins, cyclin D1 and p27, were investigated. As expected from our data on the cell cycle arresting effects of NVP-AEW541, a downregulation of cell cycle promoting cyclin D1 was observed, while on the other hand the cyclin-dependent kinase inhibitor, p27, was upregulated (Fig. 8B).

Discussion

Although control of hypersecretion syndromes can be achieved in gastrointestinal NE tumour disease by somatostatin analogues or IFN α , systemic treatment options to inhibit growth and spread of metastatic neuroendocrine tumours are still unsatisfactory.

In many human tumours (e.g. breast, colon, stomach) the insulin-like growth factor I receptor is overexpressed (Ouban *et al.* 2003). Together with its ligand IGF-I, tumour growth is stimulated by either autocrine or paracrine means (Tricoli *et al.* 1986, Cullen *et al.* 1990).

In this study, we provide evidence that inhibition of IGF-1R tyrosine kinase activity may be a promising approach for novel treatment strategies of NET disease. The specific IGF-1R tyrosine kinase inhibitor NVP-AEW541 time- and dose-dependently reduced the growth of NET cells by almost 100%. Moreover, our studies on combining NVP-AEW541 with cytostatics or with the HMG-CoA reductase inhibitor, fluvastatin, yielded additive anti-proliferative effects and qualify NVP-AEW541 for combination therapies.

The growth pattern of NE gastrointestinal tumours exhibits a wide spectrum ranging from very slow- to fast-growing, aggressive types of tumours (Öberg 1994). In view of this particular background, it was important to check whether NVP-AEW541 was equally capable of inhibiting NE tumours with different growth patterns. Therefore, we performed

Figure 7 NVP-AEW541 induced LDH release. LDH release into the supernatant of CM (A) and BON (B) cells was determined after 8 and 24 h incubation with rising concentrations of NVP-AEW541. No significant LDH release was observed during this period, indicating that NVP-AEW541 does not induce immediate cytotoxicity. Data of three independent experiments $\pm$ S.E.M. for each cell line are shown.

our studies in a carcinoid and in an insulinoma cell line, which exhibit different growth patterns. As a model for fast-growing NET cells, we chose the human insulinoma cell line CM with a doubling rate of less than one day (21 ± 1 h), while more slow-growing cells were represented by pancreatic carcinoid BON cells, which had a doubling rate of approximately twice the time (34 ± 4 h).

Although NVP-AEW541 effectively inhibited tumour growth both in CM insulinoma and BON carcinoid cells, differences concerning the signalling pathways involved became apparent. The concentration needed to induce half-maximal anti-neoplastic

effects was significantly higher in BON cells (IC_{50} : $6.6 \pm 0.6 \mu M$) than in CM cells (IC_{50} : $3.3 \pm 0.3 \mu M$).

The IGF-1R-specificity of NVP-AEW541-induced growth inhibition of either cell line was underlined by proliferation studies in serum-free medium, in which the addition of IGF-I served as the only growth-stimulating factor in the medium. Under these conditions, NVP-AEW541 also potently inhibited the proliferation of CM and BON cells in a dose-dependent manner.

However, it has been argued that high NVP-AEW541 concentrations may also inhibit the closely related InsR — especially if InsR is abundantly expressed — leading to a loss of relative IGF-1R overexpression that often occurs in tumour cells. Moreover, high IGF-I concentrations (>100 ng/ml) may also activate the InsR, thereby contributing to the growth of NET cells. However, our data on InsR and IGF-1R expression in NET cells revealed only a very weak expression of InsR, while IGF-1R was abundantly expressed in both cell lines. Moreover, the use of 100 ng/ml IGF-I for growth stimulation of NET cells in serum-free experiments appears to be below the concentration needed for significant activation of InsR. Zaka *et al.* (2005) recently demonstrated that 1 μ g/ml IGF-I was necessary to significantly phosphorylate the InsR, whereas 100 ng/ml IGF-I evoked a comparatively weak InsR activation. Thus, the anti-neoplastic effects of NVP-AEW541 on NET cells are basically due to an inhibition of IGF-1R signalling, although the marginal effects of an additional InsR inhibition cannot be completely excluded.

NVP-AEW541 was recently tested in non-neuroendocrine tumour cells with an anti-proliferative potency that is comparable to our findings on NET cells. Scotlandi and coworkers (2005) reported on half-maximal growth inhibitory effects of NVP-AEW541 in osteosarcoma cells at concentrations ranging between 1 and 6 μM .

Monotherapy is not always a successful treatment strategy as resistance or compensatory effects sometimes counterbalance a specifically blocked signalling pathway, arguing for combination treatment approaches. In this respect, we were interested in the additive anti-proliferative potency of NVP-AEW541 plus doxorubicin or NVP-AEW541 plus 5-FU, both being established cytostatic drugs for the treatment of NET disease (Öberg 2001). Co-application of doxorubicin and NVP-AEW541 resulted in slightly over-additive anti-proliferative effects in CM insulinoma cells. The observed additive effects are in accordance with the previous findings of Benini *et al.* (2001) who showed that blockade of IGF-1R signalling

Figure 8 NVP-AEW541 affects phosphorylation of ERK1/2 and expression of the apoptosis- and cell cycle-relevant proteins BAX, Bcl-2, cyclin D1 and p27. (A) Serum-starved BON cells were pretreated with NVP-AEW541 for 1 h and subsequently stimulated with IGF-I (100 ng/ml) for 15 min. The amount of phosphorylation of ERK1/2 was analysed by Western blotting. NVP-AEW541 induced a dose-dependent decrease in phosphorylated ERK1/2. Total ERK1/2 expression was not affected. (B) BON cells were incubated with NVP-AEW541 for 48 h. Expression patterns of apoptosis relevant proteins were monitored by Western blotting. Compared with untreated cells, NVP-AEW541 time-dependently downregulated anti-apoptotic Bcl-2, while pro-apoptotic BAX was upregulated. Moreover, cell cycle promoting cyclin D1 was downregulated, while the cell cycle arresting p27 was upregulated by NVP-AEW541. Representative tracings from three independent experiments are depicted.

by a monoclonal IGF-1R antibody increased the anti-tumour activity of doxorubicin in bone tumours. In this context, we additionally investigated the effects of dual-targeting the IGF-1R by NVP-AEW541 and a monoclonal IGF-1R antibody (Ab-01; Abcam, Cambridge, UK), which resulted in additive anti-proliferative effects in both BON and CM cells (unpublished observations). The potency of dual-targeting the IGF-1R by antibodies and TK inhibitors appears to be an auspicious approach for NET treatment and will be explored in detail in a forthcoming study. Dual-targeting of the epidermal growth factor receptor (EGFR) by the EGFR-TK inhibitor, gefitinib, in combination with the monoclonal EGFR antibody, cetuximab, has recently been shown to be very effective in enhancing EGFR-based cancer treatment (Matar *et al.* 2004). Together with our

preliminary observations, these findings indicate that dual-targeting of growth factor receptor signalling may generally be an interesting approach for future cancer treatment strategies and will be pursued in detail.

Recently, 5-FU has been demonstrated to cause only weak anti-proliferative effects in pancreatic neuroendocrine tumour cells. Thus, we were interested in exploring a possible anti-proliferative enhancement of 5-FU by NVP-AEW541. However, no enhancement could be observed in pancreatic neuroendocrine BON tumour cells. Interestingly, our findings are in line with recent results of Lankat-Buttgereit and colleagues (2005), who also failed to enhance the anti-proliferative efficacy of 5-FU in BON cells. In this study, 5-FU was combined with high-dose imatinib, which inhibited cellular tyrosine kinase activity (Lankat-Buttgereit *et al.* 2005).

Drug resistance is one of the major problems of chemotherapy. Potential mechanisms of drug resistance include the activation of the Ras/Raf/Mek/ERK signal transduction cascade and the increase in cholesterol levels in cancer cells, both being controlled by isoprenoids (Jakobisiak & Golab 2003). The production of isoprenoids is catalysed by HMG-CoA reductase, which may, therefore, be a rational molecular target for innovative anti-neoplastic treatment of NET disease. Fluvastatin is an effective inhibitor of HMG-CoA reductase and has already been shown to inhibit tumour cell growth (Paragh *et al.* 2003).

HMG-CoA reductase inhibitors disrupt cellular processes by the depletion of isoprenoids and dolichol. Insulin-like growth factor signalling appears particularly prone to such disruption as intracellular receptor processing requires dolichol for correct N-glycosylation, whereas downstream signalling through Ras requires the appropriate prenylation (farnesol) (Siddals *et al.* 2004). Thus, combination treatment for simultaneous inhibition of IGFR and HMG-CoA reductase signalling may lead to enhanced anti-tumour efficacy. Accordingly, we here demonstrate the anti-neoplastic effect of fluvastatin alone and in combination with NVP-AEW541 in gastrointestinal neuroendocrine tumour cells. In line with the IGFR-interfering effects of HMG-CoA reductase inhibition, fluvastatin additively enhanced the anti-proliferative effect of NVP-AEW541. Hence, combining NVP-AEW541 and HMG-CoA reductase inhibition may also be a promising approach for dual-targeting treatment strategies in NET disease.

To further characterize the underlying mechanisms of NVP-AEW541's anti-neoplastic action, we performed cell cycle analysis. In both CM and BON cells, a pronounced cell cycle arrest in the G₀/G₁ phase was observed after treatment with sub-IC₅₀ concentrations of NVP-AEW541. Interestingly, higher NVP-AEW541 concentrations did not significantly increase the cell cycle arresting effects. Our data suggest that NVP-AEW541 acts at the G₁/S checkpoint. Western blotting confirmed these findings at the molecular level, as we showed that NVP-AEW541 induced time-dependent downregulation of the cell cycle promotor, cyclin D1, while its antagonist, p27, was upregulated upon NVP-AEW541 treatment. The findings are in accordance with the studies of von Wichert and co-workers (2005) who showed that IGF-1R-induced cell cycle modulation is associated with changes in the expression pattern of these two cell cycle regulators in neuroendocrine tumour cells.

Cell cycle arrest by inhibition of the IGF/IGFR signalling system at the G₁/S checkpoint had

previously been described for non-NE tumours such as colon, prostate, breast or lung cancer (Baserga 1994, Mitsiades *et al.* 2004). Several cancers are known to be very sensitive to growth factors, many of them producing and releasing growth factors for autostimulation. This has also been suggested for NE gastrointestinal tumours in which self-produced IGF may autostimulate the IGFR pathway, thereby promoting tumour cell growth (Nilsson *et al.* 1993, 1995), further underlining the rationale of IGF-receptor inhibition as a promising target for effective tumour growth control.

Based on findings with other growth factor receptor TK inhibitors (Höpfner *et al.* 2003, Huether *et al.* 2005), we hypothesized that the anti-neoplastic effect of NVP-AEW541 was not due solely to cell cycle arrest. Confirming this notion, we discovered an increase in apoptosis-specific changes after treatment with NVP-AEW541. While the induction of apoptosis is a well-known effect occurring upon inhibition of IGFR signalling (Ellouk-Achard *et al.* 1998, Baserga *et al.* 2003, LeRoith & Roberts 2003, Camirand & Pollak 2004), the underlying mechanisms have been poorly characterized. Our results suggest that activation of caspase-3 is involved in NVP-AEW541-induced apoptosis of NET cells. Moreover, we could demonstrate that NVP-AEW541 treatment led to a shift in the equilibrium of the two predominant apoptosis-related proteins BAX and Bcl-2 (Heiser *et al.* 2004). Pro-apoptotic BAX was upregulated by NVP-AEW541, whereas the expression of anti-apoptotic Bcl-2 decreased. NVP-AEW541-induced membrane blebbing, representing an early event in the apoptotic signalling cascade, was observed after 24 h of incubation with sub-IC₅₀ concentrations of NVP-AEW541 (1 µM). The low concentrations that were required to activate the apoptotic machinery in NET cells further strengthens the interpretation that apoptosis induction by NVP-AEW541 is a specific effect contributing to the overall anti-neoplastic effects of the compound. Another remarkable feature of NVP-AEW541's anti-proliferative effect in NET cells was that the drug did not exhibit unspecific cytotoxicity, which further demonstrated the specific mode of action of the drug.

BON cells are known to secrete IGF-I. Moreover, they were shown to exhibit an autocrine-IGF loop, which is essential for their proliferation and which leads to high basal MAP kinase activity even under serum-starved culture conditions (von Wichert *et al.* 2000). In accordance with the findings of von Wichert and coworkers, we also found relatively high basal ERK1/2-activity of serum-starved BON cells. Incubation with NVP-AEW541 dose-dependently decreased

tion with NVP-AEW541 dose-dependently decreased the amount of phosphorylated ERK1/2, which thus appears to be a specific effect of IGF-1R tyrosine kinase inhibition by NVP-AEW541.

Primary cell cultures of human neuroendocrine cancers were established as a tool to design a rational individual medical treatment of an individual patient. The primary goal was to study NVP-AEW541's anti-neoplastic potency in a bench-to-bedside approach, as permanent cell lines may represent well suited but nevertheless non-representative models of neuroendocrine cancers. Moreover, chemosensitivity testing of primary cultures was performed to establish a new method for predicting the response of an individual patient to a certain drug. Attempts to predict individual responses have already been undertaken for breast cancer (Shukla *et al.* 2005) and colorectal cancer (Mori *et al.* 2003). Such an approach may pave the way to an individualized medical treatment of cancer patients. All four primary cell cultures were shown to be sensitive to NVP-AEW541 treatment, underlining the conclusion that NVP-AEW541 is an auspicious compound for future neuroendocrine cancer treatment.

To conclude, our study provides the first evidence that the IGFR-TK inhibitor, NVP-AEW541, potently inhibits the growth of human gastrointestinal neuroendocrine tumour cells by inducing both cell cycle arrest and apoptosis without eliciting unspecific cytotoxicity. Moreover, the compound is well suited for combination treatment approaches and may thus be a promising agent for an IGFR-targeted treatment strategy in NET disease. It merits further elucidation in future clinical trials.

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