Matuzumab Short-Term Therapy in Experimental Pancreatic Cancer: Prolonged Antitumor Activity in Combination with Gemcitabine

Axel Kleespies,1 Ivan Ischenko,1 Martin E. Eichhorn,1 Hendrik Seeliger,1 Christiane Amendt,2 Ole Mantell,3 Karl-Walter Jauch,1 and Christiane J. Bruns3

Abstract

Purpose: The epidermal growth factor receptor ErbB-1 is commonly expressed in pancreatic cancer and ErbB-1 targeting has shown promising results. We wanted to evaluate matuzumab (EMD72000), a fully humanized ErbB-1–specific monoclonal antibody in combination with gemcitabine in experimental pancreatic cancer.

Experimental Design: Using the human pancreatic cancer cell line L3.6pl, we investigated matuzumab in vitro and in vivo. ErbB-1 phosphorylation and downstream pathway activation were evaluated by Western blot. Proliferation and migration assays and fluorescence-activated cell sorting analysis were done. For in vivo studies, we used an orthotopic nude mice model in which 40 mg/kg of matuzumab ± 100 mg/kg of gemcitabine were administered twice weekly. Different treatment durations (7, 14, 21, and 25 days) and varying time points of treatment initiation (days 8, 15, 22, and 29) were evaluated. Ki67, CD31, and phosphorylated p44/42 mitogen-activated protein kinase (MAPK) immunohistochemistry were done.

Results: ErbB-1 phosphorylation and downstream MAPK and AKT signaling were significantly reduced by matuzumab. Matuzumab significantly inhibited proliferation and migration in vitro, and induced tumor cell apoptosis in a dose-dependent manner. Matuzumab therapy significantly lowered tumor volume in vivo, reduced lymph node and liver metastases, and decreased microvessel density and tumor cell proliferation. These effects were significantly enhanced when gemcitabine was added. A significant and prolonged antitumor activity was even evident with short-term therapy (7 days) and with a late onset of therapy (day 22 after tumor cell injection).

Conclusions: Matuzumab is an effective agent with long-lasting antiproliferative, proapoptotic, antiangiogenic, and antimitastatic activity in human pancreatic cancer models. These effects might be potentiated by gemcitabine.

For >80% of pancreatic cancer patients, palliative treatment protocols represent the only reasonable therapeutic option (1, 2). Gemcitabine is the current standard drug for cytotoxic therapy of advanced and metastatic pancreatic cancer (3). However, the results obtained with gemcitabine monotherapy have been modest and combination regimens with other cytotoxic drugs have rarely translated into survival benefit (3–5). For that reason, there is a great demand for the development of new effective drugs and novel therapeutic strategies (6). Among those strategies, drugs that target cancerspecific molecular defects hold the greatest promise for the near future (targeted therapy; refs. 3, 7–9).

Pancreatic cancer is characterized by genetic and regulatory alterations including several receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) ErbB-1 (8, 10). Many features of the malignant phenotype, such as increased cell proliferation, cell survival, and angiogenesis, are associated with signaling networks that involve ErbB-1 (11). ErbB-1 activation leads to elevated recruitment of downstream mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K). The PI3K/AKT pathway suppresses apoptosis by activating the AKT antiapoptotic protein kinase, whereas MAPK pathway activation is associated with tumor cell proliferation (8). ErbB-1 expression was detected in up to 90% of pancreatic cancers (7, 12, 13) and coexpression of ErbB-1 and of at least one of its ligands (epidermal growth factor, transforming growth factor-α) has been shown to predict poor prognosis in human pancreatic cancer (12, 14). ErbB-1 signaling may be inhibited by monoclonal antibodies (mAb) designed to block the extracellular ligand–binding domain of ErbB-1 (15). We have shown previously that cetuximab, a chimeric (human-mouse) mAb directed against ErbB-1, inhibits tumor growth and metastases of human pancreatic cancer xenografts in nude mice (16). Matuzumab
**Translational Relevance**

Gemcitabine is the current standard drug for cytotoxic therapy of pancreatic cancer. However, the results obtained with gemcitabine monotherapy have been modest and combination regimens with other cytotoxic drugs have rarely translated into survival benefit. ErbB-1 represents a viable target in the treatment of pancreatic cancer and ErbB-1 signaling may be inhibited by monoclonal antibodies. Matuzumab is a new fully humanized anti–ErbB-1 monoclonal antibody, which seems to offer immunologic and pharmacokinetic advantages over human-mouse chimeras. Matuzumab has recently entered early clinical trials. However, preclinical data supporting the antitumor efficacy of matuzumab are very rare. The present report is the first to publish preclinical studies of matuzumab and standard gemcitabine therapy in experimental pancreatic cancer. We wanted to highlight the biological mechanisms which might be responsible for the clinical efficacy of matuzumab as well as to evaluate the different treatment regimens in combination with gemcitabine. Our studies could show that matuzumab significantly blocks ErbB-1 downstream signaling via MAPK and PI3K/AKT pathways, and reveals significant antiproliferative, antiangiogenic, antimetastatic, and proapoptotic activity in combination with gemcitabine. Combination therapy was superior to gemcitabine monotherapy because long-lasting antitumor activity was evident even after short-term, ultra-short-term, and late onset of therapy. We believe that this article may be of particular interest to the readers of *Clinical Cancer Research* because our data are translational and clearly show a new perspective in future pancreatic cancer therapy.

**Materials and Methods**

**Cell culture.** For *in vitro* and *in vivo* testing of matuzumab (±gemcitabine), the highly metastatic human pancreatic cancer cell line L3.6pl was used (23). L3.6pl cells were cultured in DMEM, supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, a 2-fold vitamin solution (Life Technologies, Inc.), and a penicillin-streptomycin mixture (Flow Laboratories) to constitute complete DMEM. Monolayer cultures were maintained on plastic and incubated in a mixture of 5% CO2 and 95% oxygen at 37°C. The culture was free of Mycoplasma and pathogenic murine viruses (assayed by MA Bioproducts), and was maintained for no longer than 12 weeks after recovery from frozen stocks.

**Compounds.** Matuzumab (formerly EMD72000) was kindly provided by Merck KGAA (Darmstadt, Germany). Gemcitabine (Gemzar, Eli Lilly and Co.) and human recombinant EGF (R&D Systems) were obtained commercially. Antibodies and staining reagents for Western blotting and immunohistochemistry were purchased as follows. For Western blotting: rabbit anti-human EGF, rabbit anti-human phospho-EGFR (Yyr1068), rabbit anti-human phospho-p44/42 MAPK, rabbit anti-human phospho-p44/42 MAPK (Thr202/Yyr204), rabbit anti-human AKT, rabbit anti-human phospho-AKT (Ser473), Cell Signaling/New England Biolabs, mouse anti-human β-actin (Sigma-Aldrich Chemie GmbH), and goat anti-rabbit and goat anti-mouse horseradish peroxidase secondary antibodies (Dako A/S). For immunohistochemistry: rat anti-mouse CD31/PECAM-1 (PharMingen), rabbit anti-human Ki67 (Zymed Laboratories, Inc.), rabbit anti-human phospho-p44/42 MAPK (Thr202/Yyr204; Cell Signaling/New England Biolabs), peroxidasen conjugated goat anti-rat IgG secondary antibody (Jackson Immunoresearch Laboratories), and biotinylated goat anti-rabbit IgG secondary antibody (BA-1000, Vector Laboratories, Inc.). All antibodies were used according to the manufacturer’s instructions. Other consumables used included ABC detection kit (Vector Laboratories, Inc.), DAB Map kit 760-126 (Ventana Medical Systems, Inc.), Gill’s hematoxylin (Sigma Chemical, Co.), optimum cutting temperature compound (Miles, Inc.), positively charged superfrost plus slides (Menzel-Gläser), and VectaMount mounting medium (Vector Laboratories, Inc.).

**Western blotting for analysis of ErbB-1, MAPK, and AKT phosphorylation in vitro.** To investigate the molecular mechanisms responsible for the potential biological effect of matuzumab in combination with gemcitabine, we analyzed the effect of both agents on the expression and phosphorylation of ErbB-1, as well as on the main steps of downstream MAPK and PI3K/AKT pathways. Western blotting was done for the detection of total and phosphorylated ErbB-1, total and phosphorylated p44/42 MAPK, and total and phosphorylated AKT. Briefly, nonconfluent L3.6pl cells were starved overnight in serum-free DMEM followed by treatment with matuzumab, gemcitabine, or a combination of both drugs for 1 h. The cells were further stimulated with 50 ng/ml of EGF for 10 min, washed with ice-cold PBS, and resuspended in ice-cold radioimmunoprecipitation assay buffer supplemented with Complete Mini EDTA–free Protease Inhibitor Cocktail (Roche) to a final concentration of –10−6 to 10−9 cells/ml. Finally, the cells were incubated on ice for 10 min and centrifuged at 14,000 × g at 4°C for 10 min. An equal amount of protein was run on polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore), and detected using an enhanced chemiluminescence system (Amersham).

**Cell proliferation assay.** To analyze the effect of matuzumab in combination with gemcitabine on EGF-driven proliferation of L3.6pl cells in vitro, a TACS 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation and viability assay was done (R&D Systems). L3.6pl cells (105) were cultured in 96-well plates in serum-reduced EGF-containing (50 ng/ml) DMEM in the presence of matuzumab at different concentrations (p44/42 MAPK IC50 (0.01-100 ng/ml), gemcitabine IC50 (50 ng/ml), or a combination of both agents (24). After 48 h, the cells were washed with PBS, the MTT reagents were added according to

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**EMD72000; Merck** is a novel, fully humanized mAb which binds to ErbB-1 with high affinity and specificity (17), and which seems to offer immunologic and pharmacokinetic advantages over human-mouse chimeras. A longer half-life of this agent might be advantageous, and one administration of matuzumab every 2 to 3 weeks was proposed to be sufficient in humans (18). Matuzumab is currently under clinical investigation in patients with advanced stage solid cancer, such as non–small-cell lung cancer and ovarian cancer (19–21), and was tolerated well in patients with pancreatic cancer who received an additional standard gemcitabine therapy (22). However, in contrast to cetuximab, which has been broadly studied in preclinical models, experimental data supporting the antitumor efficacy of matuzumab are very rare.

The aim of this study is to assess the *in vivo* effects of matuzumab in an orthotopic pancreatic tumor model. Different treatment schedules were chosen to evaluate the effect of multiple-dosing, short-term treatment, late onset of treatment, and additional gemcitabine therapy. Furthermore, we examined the antiproliferative, proapoptotic, antiangiogenic, and antimetastatic activities of matuzumab and its capacity to inhibit ligand-dependent ErbB-1 phosphorylation and subsequent MAPK and AKT activation *in vitro*.**
the manufacturer’s recommendations, and the absorbance was measured at 570 nm using an ELISA plate reader. The IC_{50} was defined as the drug concentration that causes a 50% inhibition of cell proliferation. Mean values were calculated from two independent experiments.

**Cell migration assay.** To assess the ability of matuzumab to inhibit the in vitro migration activity of L3.6pl cells, we did a modified Boyden chamber assay. Supernatant cells (10^5 per well) were seeded into the upper well of a chamber system (Becton Dickinson Falcon cell culture insert; BD Biosciences) on a human fibronectin-coated (Sigma-Aldrich Chemicals) polyethylene terephthalate membrane with 8-μm pores. As a chemoattractant, human recombinant EGF was added at 50 ng/mL in serum-reduced DMEM into the lower well of the chamber. Inhibition of EGF-induced chemotaxis was assessed after adding matuzumab at 10 or 100 μg/mL. Migration through the membrane was assessed after 12 h of incubation at 37°C by fixing, H&E staining, and counting the migrated cells in five random fields at ×100 magnification. The average of duplicate inserts was obtained.

**Determination of apoptosis by fluorescence-activated cell sorting analysis.** We did a propidium iodide staining for cell cycle analysis to evaluate if the combination of matuzumab and gemcitabine in vitro would induce the apoptosis of L3.6pl cells under EGF-dependent cell culture conditions. Nonconfluent L3.6pl cells were plated into six-well plates, starved overnight in serum-reduced DMEM and further cultured in the presence of matuzumab (10 or 100 μg/mL), gemcitabine IC_{50} (50 ng/mL), or the combination of both agents in the presence of EGF (50 ng/mL). After 24 h, the cells were collected and suspended in a Nicoletti buffer [0.1% sodium citrate (pH 7.4), 0.1% Triton X-100, and 50 μg/mL propidium iodide]. DNA content present in the resulting nuclei was determined on a fluorescence-activated cell sorter (FACS; Becton Dickinson GmbH). The fraction of L3.6pl cells in the sub—G_0/G_1 phase of cell cycle was considered as apoptotic. All experiments were replicated thrice.

**Orthotopic tumor model.** Male athymic nude mice (BALB/c nu/nu) were purchased from Charles River Laboratories, housed and maintained in laminar flow cabinets under specific pathogen-free conditions, and used in accordance with institutional and governmental guidelines at 8 to 12 weeks of age. L3.6pl cells were harvested from subconfluent cultures by treatment with 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in HBSS. Only single-cell suspensions with >90% viability were used for pancreatic injections. To induce pancreatic tumors, tumor cells were injected orthotopically into the pancreas of nude mice as described previously using 1 × 10^6 viable L3.6pl cells suspended in 0.2 mL PBS (23, 24).

**Multiple-dosing treatment in vivo.** The first experimental setting included a total of 41 animals which were orthotopically injected with L3.6pl cells on day 0. Eight days after tumor cell injection four mice were sacrificed and small established primary tumors, with a median tumor volume of 27 mm³ (range, 22-48 mm³), but no liver nor lymph node metastases were found. Drug treatment of the remaining animals was started at the same day. Four groups of animals received twice weekly i.p. injections of either matuzumab (40 mg/kg i.p.; n = 10), gemcitabine (100 mg/kg i.p.; n = 9), a combination of both agents at the same doses (n = 10), or PBS (n = 8). The side effects of the treatment were evaluated by monitoring body weight and lethality. All animals were sacrificed on day 33 (25 days after initiation of therapy) when control animals became moribund. Pancreatic tumors were removed, weighed, and one part of each tumor was fixed in formalin and embedded in paraffin for immunohistochemical evaluation. The other parts of the primary tumors were embedded in optimum cutting temperature compound (Miles), snap-frozen in liquid nitrogen, and prepared for frozen sectioning. Liver and lymph node metastases and peritoneal carcinomatosis were assessed macroscopically and confirmed histologically by H&E staining.

**Cytotoxicity of activated MAPK in vivo.** To evaluate, if in vivo application of matuzumab would also inhibit the ErbB-1 downstream signaling via the MAPK pathway, we additionally examined the phosphorylation of p44/42 MAPK in primary tumors after multiple-dosing experiments (see above). Activated MAPK (phospho-p44/42 MAPK) was determined by immunohistochemistry using paraffin embedded parts of the tumors and the staining instrument Discovery with the DAB Map kit 760-124 (both from Ventana Medical Systems, Inc.). Sections of 4 to 6 μm were mounted on positively charged superfrost slides, deparaffinized and heated in Tris-EDTA buffer for 30 min (pH 8.0). Endogenous peroxidase was blocked by 1% hydrogen peroxide, and sections were incubated with the primary anti–phospho-p44/42 antibody for 32 min at 37°C (1:50). Positive reaction was visualized by incubation with a biotinylated secondary antibody (8 min, 37°C, 1:500) and application of horseradish peroxidase–conjugated streptavidin and 3,3'-diaminobenzidine (DAB Map kit). Subsequently, the intensity of the immunohistochemical reaction was quantified by using an image analysis system for densitometric evaluation (Quantimet 500 Qwin, version V02.00B, Leica Microsystems). The absorbance of positively stained areas per tumor was measured in 30 randomly chosen fields, each 0.047 mm², using an ×20 objective. Due to the absence of counterstaining, the absorbance could be solely attributed to the product of the immunohistochemical reaction. The image analysis system was calibrated with an absorbance standard of 0.1, 0.3, 0.6, and 0.9. For microscopic pictures, serial sections were counterstained with hematoxylin and mounted.

**Immunohistochemistry for Ki67 and CD31.** Evaluation of tumor cell proliferation in primary tumors was done using Ki67 staining as described previously (24). Concisely, paraffin sections of 4 to 6 μm were deparaffinized in xylene, stepwise rehydrated in ethanol and PBS, overlaid with 10 mmol/L of citrate buffer (pH 6.0) and microwaved for 15 min at 750 W to achieve Ki67 antigen demasking. After cooling down, the slides were rinsed with PBS, and nonspecific binding sites were blocked with 5% bovine serum albumin in PBS. After another washing step with PBS, the primary antibody was applied at a dilution of 1:75, and the slides were incubated for 2 h at room temperature. Then, samples were incubated with the biotinylated secondary antibody (1:200) for 1 h at room temperature, and a standard streptavidin-biotin method with 3,3'-diaminobenzidine as chromogen was used for highlighting Ki67-positive cells. Slides were counterstained with hematoxylin and mounted. Sections were analyzed microscopically, and the number of positive staining cells were counted. Vascularization of primary tumors was analyzed by CD31 immunohistochemistry on frozen tissue. Sections of 8 to 9 μm were air-dried, fixed in cold acetone for 5 min, acetone/chloroform for 5 min, acetone for 5 min, and then washed in PBS. After that, CD31 immunohistochemical staining was done as described previously, and microvessel density was assessed (25). For all immunohistochemical studies (Ki67, CD31), five sections of each tumor were assessed microscopically. The average numbers of positive staining cells (Ki67) and of positive staining microvessels (CD31) were calculated by analysis of 7 to 10 high-power fields per section (0.159 mm²). Microscopic assessment was done by two independent observers who were blinded to all experimental data.

**Short-term therapy.** A second animal experiment was conducted to evaluate the effect of different treatment durations in the same experimental setting (short-term therapy). Seven groups of nude mice (n = 8-10) were injected orthotopically with 1 × 10^6 L3.6pl cells. Four groups received a combination therapy of matuzumab and gemcitabine twice weekly after primary tumors were established (40 and 100 mg/kg i.p., respectively). As in the first experiment, therapy started at day 8 after tumor cell injection but was continued for either 7, 14, 21, or 25 days, respectively, depending on the treatment group (see Fig. 6A). For comparison, another three groups of animals were treated with only gemcitabine, matuzumab, or PBS (control) for 25 days. Again, all animals were sacrificed at day 33 irrespective of their treatment group. Pancreatic tumors were removed, weighed, and histologically confirmed by H&E staining.

**Late onset of therapy.** To assess therapeutic effects of matuzumab in more advanced disease, we conducted additional experiments were
the start of treatment was postponed to later time points (late onset therapy). Another seven groups of animals \((n = 8-10)\) were injected with \(1 \times 10^6\) L3.6pl cells at day 0. Combination treatment with matuzumab and gemcitabine (40 and 100 mg/kg i.p., twice weekly) was started on days 8, 15, 22 or 29, respectively, depending on the treatment group (Fig. 6C). The three remaining groups received only gemcitabine, matuzumab, or PBS starting on day 8. All mice were sacrificed at day 33 and pancreatic tumors were removed, weighed, and stained.

Statistical analysis. Data are presented as means \(\pm SD\). In vitro studies, body weight, pancreatic tumor weight, immunohistochemical quantification of Ki67, CD31 and p44/42 MAPK staining were compared using a one-way ANOVA with a Student-Newman-Keuls multiple comparisons test (InStat 3.0 Statistical Software, GraphPad Software). The relative rates of liver and lymph node metastases within groups were compared by Fisher’s exact test. For all analyses, \(P < 0.05\) was considered to be significant. All \(P\) values were two-tailed.

Results

Matuzumab inhibits ErbB-1, MAPK, and AKT phosphorylation in vitro. Matuzumab treatment of L3.6pl cells in vitro blocked EGF-induced activation of ErbB-1 in a dose-dependent manner (Fig. 1A). Matuzumab inhibited ErbB-1 phosphorylation (phospho-EGFR) at concentrations of 10 \(\mu g/mL\) without affecting total ErbB-1 expression. However, the maximum inhibitory effect occurred at 100 \(\mu g/mL\). Downstream signaling of ErbB-1 was also inhibited by matuzumab in vitro. Phosphorylation of p44/42 MAPK (phospho-p44/42 MAPK) was inhibited at doses of 10 to 100 \(\mu g/mL\), whereas total expression of p44/42 MAPK remained unchanged (Fig. 1A). Phosphorylation of AKT (phospho-AKT) was also moderately inhibited by matuzumab at doses of 10 g/mL and 100 \(\mu g/mL\) without alteration of total AKT expression (Fig. 1B). However, the combination treatment with gemcitabine (50 ng/mL) resulted in even stronger inhibition of EGF-induced receptor signaling. Particularly, downstream activation of p44/42 MAPK (phospho-p44/42 MAPK) was diminished by the combination of matuzumab and gemcitabine (Fig. 1B).

Matuzumab inhibits cell proliferation in vitro. EGF stimulation of L3.6pl cells significantly increased tumor cell proliferation in vitro (Fig. 2A). A significant antiproliferative activity of matuzumab on EGF-stimulated cells could be observed at concentrations of 0.01 \(\mu g/mL\). Complete inhibition of EGF stimulation was achieved with 0.1 \(\mu g/mL\) of matuzumab and maximum antiproliferative effects were obtained at doses of 100 \(\mu g/mL\) (IC\(_{50}\) = 10 \(\mu g/mL\)). However, when gemcitabine was added at a dose of 50 ng/mL, antiproliferative effects were even stronger and significant differences were observed between treatment with matuzumab alone and in combination with gemcitabine (Fig. 2A).

Matuzumab inhibits cell migration in vitro. As migration of tumor cells is an integral part of their metastatic activity, we investigated the migratory activity of EGF-stimulated L3.6pl cells following treatment with matuzumab. Indeed, 12 hours of treatment with matuzumab resulted in a significant reduction of the migratory activity of tumor cells at concentrations of 10 and 100 \(\mu g/mL\) (Fig. 2B).

Matuzumab and gemcitabine induce apoptosis and cell cycle arrest in vitro. We next studied whether receptor blockade
and subsequent inhibition of downstream signaling by matuzumab and gemcitabine might result in proapoptotic effects in vitro. As detected by propidium iodide staining for cell cycle, the percentage of the cell population undergoing apoptosis was moderately increased after gemcitabine monotherapy (Fig. 3A). However, matuzumab significantly induced apoptosis in a dose-dependent manner, and the combination of both agents resulted in an even stronger tumor cell apoptosis (Fig. 3A). Furthermore, our results clearly show a therapy-induced cell cycle arrest with most impressive effects after combination therapy. Cell cycle arrest was considered as an accumulation of cells in G0-G1 phase in the absence of G2-M and S phases of cell cycle (Fig. 3B).

Matuzumab and gemcitabine inhibit tumor growth and metastatic spread in vivo. All animals developed macroscopic pancreatic tumors after orthotopic tumor cell injection. Multiple-dosing of matuzumab seemed to be well-tolerated, and assessment of body weight showed no significant differences between all treatment groups (Table 1). A significant reduction of primary tumor weight was found in all treatment groups compared with controls (Fig. 4A; Table 1). Matuzumab and gemcitabine inhibited tumor growth to a comparable extent (by 43% and 37%, respectively). However, tumor growth inhibition was strongest with a combination therapy of matuzumab and gemcitabine, which resulted in 63% tumor shrinkage compared with controls (control, 1,650 mg; combination therapy, 614 mg; P < 0.01; Table 1).

Matuzumab reduces microvessel density and proliferation index in vivo. No reduction in microvessel density (CD31) or proliferation index (Ki67) was found in gemcitabine-treated tumors when compared with controls (Fig. 5B and C). However, compared with gemcitabine treatment and controls, we found a significantly compromised tumor cell proliferation, and a significantly reduced vascularization in matuzumab-treated tumors and in tumors treated with the combination of both agents. The combination of matuzumab and gemcitabine was even more antiangiogenic and antiproliferative than matuzumab alone (Fig. 5B and C).

Matuzumab short-term therapy is effective. Even with limited treatment duration, the combination therapy of matuzumab and gemcitabine still inhibited tumor growth significantly when compared with controls (Fig. 6B). Even after
a short-term therapy (14 days) or an ultra-short-term therapy of only 7 days (two injections) treatment effects were significant. Moreover, the ultra-short-term combination regimen of only two doses of matuzumab and gemcitabine seemed to be as effective as multiple-dosing of either drug alone (Fig. 6B).

**Late onset of therapy shows antitumor activity.** A 1-, 2-, or 3-week delay of treatment initiation resulted in gradually

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**Fig. 3.** Combination of matuzumab and gemcitabine induces tumor cell apoptosis and cell cycle arrest in vitro. **A**, as detected by propidium iodide staining for the cell cycle, apoptosis of L3.6pl cells was moderately enhanced by gemcitabine but significantly increased with matuzumab or the combination of both agents in a dose-dependent manner (*, P < 0.05 and **, P < 0.001 vs. untreated cells; ###, P < 0.001 vs. gemcitabine alone; ***, P < 0.01 vs. matuzumab alone). **B**, treatment with gemcitabine and matuzumab was sufficient to cause a cell cycle arrest, as detected by an accumulation of cells in G0-G1 phase in the absence of G2-M and S phases of cell cycle (histograms show one of three representative independent experiments).
increased tumor volumes (Fig. 6D). However, the combination therapy of matuzumab and gemcitabine still inhibited tumor growth significantly in this experiment. Even late application of that therapy for large tumors at day 22 (2 weeks delay) could reduce primary tumor weights to an extent which was nearly comparable to that seen with early onset multiple-dosing therapy of gemcitabine alone (Fig. 6D).

Discussion

There is accumulating preclinical and clinical evidence that ErbB-1 represents a viable target in the treatment of pancreatic cancer. Elevated expression of ErbB-1 was detected in >90% of human pancreatic cancer samples (12) and coexpression of ErbB-1 and its ligands (EGF, transforming growth factor-α) was associated with increased tumor size, advanced clinical stage, and poor prognosis (13, 14, 26, 27). We and others could show that a combination of chemotherapeutic agents (gemcitabine, 5-fluorouracil) and cetuximab (C225, Erbitux), a chimeric mouse-human IgG 1 mAb against ErbB-1, exhibits significant antitumor activity in pancreatic cancer models (16, 28, 29). Moreover, cetuximab in combination with gemcitabine showed promising activity against advanced pancreatic cancer in a phase II clinical trial (30). However, the immunogenicity of chimeric mAbs like cetuximab remains a significant concern because sensitization to chimeric mAbs may blunt the long-term efficacy of these anticancer drugs. Recent advances in genetic engineering now allow the design of fully humanized antibodies for which the patient’s immune response directed against the murine protein is minimal. This technique has led to the development of matuzumab (EMD72000), a fully humanized IgG 1 anti–ErbB-1 mAb. Matuzumab binds competitively to ErbB-1, with higher affinity than natural growth factors. The resulting blockade inhibits ligand-induced ErbB-1 activation and should abrogate receptor-mediated downstream signaling. Even though matuzumab has now entered early clinical trials (19, 21, 22), published preclinical data on matuzumab are very rare. To the best of our knowledge, the present study is the first to publish experimental data of matuzumab in combination with gemcitabine therapy in human pancreatic cancer models.

Table 1. Inhibition of tumor growth (weight) and metastatic spread following 25 d of treatment

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Pancreatic tumors</th>
<th>Liver metastases</th>
<th>Lymph node metastases</th>
<th>Peritoneal carcinomatosis</th>
<th>Mean tumor weight (mg) ± SD</th>
<th>Mean body weight (g) ± SD</th>
</tr>
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<tr>
<td>Control (PBS)</td>
<td>8/8</td>
<td>3/8</td>
<td>8/8</td>
<td>1/8</td>
<td>1,650 ± 445</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>Gemcitabine (100 mg/kg)</td>
<td>9/9</td>
<td>3/9</td>
<td>9/9</td>
<td>0/9</td>
<td>1,032 ± 165*</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Matuzumab (40 mg/kg)</td>
<td>10/10</td>
<td>1/10</td>
<td>5/10*</td>
<td>2/10</td>
<td>935 ± 84*</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>Matuzumab + gemcitabine (40 + 100 mg/kg)</td>
<td>10/10</td>
<td>0/10*</td>
<td>2/10*</td>
<td>1/10</td>
<td>614 ± 177*</td>
<td>21 ± 2</td>
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*P < 0.01 compared with control.
†P < 0.01 compared with gemcitabine.
‡P < 0.05 compared with control.
§P < 0.05 compared with gemcitabine.
‖P < 0.05 compared with matuzumab.

Fig. 4. Macroscopic samples of pancreatic primary tumors (A) and liver metastases (B) following 25 d of treatment (33 d after orthotopic tumor cell injection).
Fig. 5. Immunohistochemistry of primary tumors following 25 d of treatment. 

A. in vivo MAPK activation: determination of activated p44/42 MAPK (phospho-p44/42 MAPK) in primary tumors. MOD, mean optical density of phospho-p44/42 MAPK staining intensity, as measured by densitometry (*, \( P < 0.05 \) vs. control; **, \( P < 0.01 \) vs. control). 

B. Ki67 expression (proliferating cells): analysis of Ki67 expression (proliferating cells); representative randomized high-power fields at \( \times 100 \) magnification = 159 mm² (*, \( P < 0.001 \) vs. control; **, \( P < 0.001 \) vs. gemcitabine; ###, \( P < 0.005 \) vs. matuzumab). 

C. CD31 staining and quantification of microvessel density: randomized high-power fields at \( \times 100 \) magnification = 159 mm² (*, \( P < 0.001 \) vs. control; **, \( P < 0.001 \) vs. gemcitabine; ###, \( P < 0.005 \) vs. matuzumab).
A well-documented signaling route of the ErbB-1 receptor is the mitogenic MAPK pathway (Ras-Raf-MEK-MAPK pathway; ref. 9). The MAPK p44/42 subsequently regulates cell transcription and has been linked to cell proliferation, survival, and chemoresistance in pancreatic cancer (31). Elevated levels of MAPK activation have been found in several tumor types (32–34), and reduced phosphorylation of p44/42 MAPK was reported after treatment with Erb-B1 inhibitors (9, 35). This specific effect could be confirmed for matuzumab by the present study. Our in vitro results suggest that matuzumab competitively inhibits EGF-induced Erb-B1 phosphorylation and MAPK activation following higher doses of the drug. Interestingly, MAPK pathway inhibition was most evident in combination with gemcitabine treatment, suggesting at least an additive antiproliferative effect of both drugs in vitro.

The PI3K/AKT signaling pathway is another pathway downstream of ErbB-1 which might be important in pancreatic cancer survival. AKT, once phosphorylated, activates various other proteins resulting in cell survival and proliferation (36). This pathway was found to be active in pancreatic cancer (37), and PI3K/AKT signaling has also been implicated in the resistance of pancreatic cancer to gemcitabine (38, 39). In our in vitro experiments, matuzumab induced moderate inhibition of AKT-phosphorylation without alteration of total AKT in vitro, and gemcitabine exhibited an additive inhibitory effect. Out of this finding, we hypothesized that besides the potential antiproliferative effects due to MAPK inhibition, a combination of matuzumab and gemcitabine might also exhibit proapoptotic activity by blocking AKT in vitro.

Indeed, as we could show by further in vitro experiments [MTT assay, FACS analysis], matuzumab and gemcitabine significantly inhibit proliferation (matuzumab IC\textsubscript{50} = 10 μg/mL), and induce tumor cell apoptosis and cell cycle arrest in human pancreatic cancer cells. Both antiproliferative and proapoptotic effects of matuzumab are dose-dependent, and the combination of both drugs might potentiate their antineoplastic activity. Those results are in obvious conflict with a recent publication by Feng et al. (40). The authors report that gemcitabine may cause phosphorylation and subsequent degradation of Erb-B1 in UMSCC cancer cells. Consequently, targeting agents that may prevent Erb-B1 degradation (e.g., Erb-B1 mAbs) might anticipate this potential mechanism of gemcitabine-induced cell death. In our in vitro experiments, however, there was no evidence that gemcitabine might cause phosphorylation or degradation of Erb-B1 in L3.6pl cells following 1 hour of treatment (Fig. 1B). Although we cannot exclude receptor degradation hours and days after gemcitabine treatment with this experiment, we have previously shown that 4 weeks of treatment with gemcitabine does not induce Erb-B1 degradation or changes in Erb-B1 phosphorylation in L3.6pl tumor xenografts (16). These findings and the promising results of our in vitro experiments suggest that Erb-B1 degradation might not be the predominant mechanism of action of gemcitabine in L3.6pl pancreatic cancer cells.

To further study the combination of matuzumab and gemcitabine in vivo, we chose an orthotopic xenograft model with a rapid tumor growth (23). Use of such a model limited the duration of treatment protocols to a couple of weeks. Consequently, we had to start treatment early, at day 8 in multiple-dosing experiments. We chose this point of treatment initiation, as primary tumors are well established at this time (tumor volume, 22-48 mm\textsuperscript{3}), but still confined to the pancreas. Thereby, we wanted to imitate the clinical situation, in which a successful therapy is still possible before subsequent metastatic spread occurs. A significant activity against the primary tumor was evident for both gemcitabine and matuzumab therapy, but maximum effects on tumor growth were seen when both agents were given simultaneously.

The antineoplastic activity of matuzumab in vivo was associated with a significant MAPK pathway inhibition, and
the combination therapy was again most effective. Downregulation of this important mitogenic pathway correlated with a significantly reduced proliferation index (Ki67), particularly in matuzumab-treated tumors. Vanhoef er et al. recently confirmed these results in a clinical setting (21). Immunohistochemical analyses in our experiments revealed that matuzumab-mediated antitumor activity was also associated with a significant reduction of microvessel density. This antiangiogenic effect of matuzumab correlates with findings on other antibodies and tyrosine kinase inhibitors which specifically block ErbB-1 receptor (16, 17, 25, 41). Exclusive gemcitabine therapy affected neither the fraction of proliferating tumor cells nor tumor vascularization, however, combination therapy with both agents was more effective than matuzumab alone. These synergistic interactions of matuzumab and gemcitabine on tumor growth and tumor angiogenesis might be partly explained by an accumulation of different antineoplastic mechanisms from both agents (9), resulting in irreparable damage of both tumor and endothelial cells, as it was shown for other ErbB-1 inhibitors (16, 25, 42).

Matuzumab has also been shown to attract immunocompetent cells by antibody-dependent cellular toxicity (ADCC) (43). As ADCC is believed to be a predominant mode of action of many anticancer mAbs, one could speculate that reduced tumor volume in vivo might be strictly due to ADCC (44). However, the therapeutic effects seen are not proven solely by the presence of ADCC. In particular, our in vitro results should be target-specific because antibody-dependent cellular toxicity requires three components, an antigen (ErbB-1), a specific antibody (matuzumab), and an Fc receptor bearing effector cell (e.g., natural killer cell). The latter was not present in vitro.

Multiple-dosing of matuzumab was also associated with a marked antitumor activity in vivo. Liver and lymph node metastases could be prevented in >50% of the animals by matuzumab administration, and this effect was not enhanced by additional treatment with gemcitabine. Those results correspond well with previous studies testing different ErbB-1 inhibitors (16, 25). To further show that the reduction in metastases was not only due to reduced cellularity, we also did a Boyden chamber assay. The migration of tumor cells was significantly impeded after treatment with matuzumab. These results suggest that matuzumab holds a specific antitumor activity and is superior to gemcitabine therapy with respect to pancreatic tumor spread.

Because of the assumed longer half-life of matuzumab compared with other ErbB-1 inhibitors, and because of matuzumab’s competitive and strong binding to the EGF receptor, this drug may exhibit a long-lasting activity even after short-term therapy (18). To prove this hypothesis with respect to human pancreatic cancer, we tested a series of additional treatment schedules in our orthotopic tumor model (Fig. 6A). The combination of gemcitabine and matuzumab, which was most effective in multiple-dosing experiments, was further analyzed for short-term therapy and showed remarkable activity regarding tumor growth inhibition. Even an ultra-short therapy of only two injections during the first week, followed by 3 weeks without any treatment, revealed about the same extent of tumor growth inhibition as full-length therapy with at least eight injections of either drug alone. Two weeks of combination therapy produced even better results than long-term gemcitabine monotherapy, and the antitumor activity was nearly comparable to that seen during a full course therapy with both agents (Fig. 6B). However, it might have been possible that the positive treatment results in these short-term experiments were only due to an early onset of treatment. Thus, the small tumor size at this time could have been more important than the prolonged activity of the drug. To exclude this potential effect, we conducted additional experiments. Treatment initiation was postponed after tumor induction, resulting in a 1-, 2-, or 3-week delay before treatment began (Fig. 6C). Thereby, we could assess the therapeutic effects of matuzumab in more advanced stages of the disease. These experiments revealed that primary tumor weight increased with later onset of treatment (Fig. 6D). However, this increase of tumor mass should not be interpreted as a treatment failure in large tumors. Our observation rather reflects a stabilization of an advanced disease. Late-onset therapy which started at day 22 (following 3 weeks without any treatment) may be also regarded as a short-term therapy of only three injections. Nevertheless, this therapy still decelerated tumor growth to a similar extent as did a full-length, early-onset therapy with gemcitabine alone.

It was shown clinically in patients with advanced ErbB-1–positive solid malignancies that treatment with matuzumab at doses of 400 to 1,600 mg/wk is well tolerated (19, 21, 22). Those doses resulted in maximum serum concentrations of 125 to 852 μg/mL, which are 10-fold greater than those required to exhibit maximum in vitro effects in our experiments (10-100 μg/mL). Therefore, we may conclude that the clinical efficacy of matuzumab might be achievable at well tolerated doses (19, 21, 22).

In conclusion, our studies confirm that matuzumab significantly blocks ligand-dependent ErbB-1 phosphorylation and constrains receptor-mediated downstream signaling in human pancreatic cancer cells. Matuzumab reveals significant anti-proliferative, antiangiogenic, antimetastatic, and proapoptotic effects in pancreatic cancer models, and its combination with gemcitabine might be superior to standard gemcitabine therapy regarding long-lasting antitumor effects and antimetastatic activity. Further clinical trials will be necessary to establish the precise therapeutic value of a matuzumab-gemcitabine combination therapy in patients with pancreatic cancer.

Disclosure of Potential Conflicts of Interest

Merck KGaA provided C.J. Bruns with EMD 72000 (Matuzumab) used in this paper; C.Amendt is employed by Merck KGaA, Germany.

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