A novel approach in the treatment of neuroendocrine gastrointestinal tumours. Targeting the epidermal growth factor receptor by gefitinib (ZD1839)

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Therapeutic options to inhibit the growth and spread of neuroendocrine (NE) gastrointestinal tumours are still limited. Since gefitinib (4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy)quinazoline), an inhibitor of epidermal growth factor receptor-sensitive tyrosine kinase (EGFR-TK), had been shown to suppress potently the growth of various non-NE tumour entities, we studied the antineoplastic potency of gefitinib in NE gastrointestinal tumour cells. In human insulinoma (CM) cells, in human pancreatic carcinoid (BON) cells and in NE tumour cells of the gut (STC-1), gefitinib induced a time- and dose-dependent growth inhibition by almost 100%. The antiproliferative potency of gefitinib correlated with the proliferation rate of the tumour cells. So the IC50 value of gefitinib was 4.7 ± 0.6 μM in the fast-growing CM cells, still 16.8 ± 0.4 μM in the moderate-growing BON cells, and up to 31.5 ± 2.5 μM in the slow-growing STC-1 cells. Similarly, the induction of apoptosis and cell-cycle arrest by gefitinib differed according to growth characteristics: fast-growing CM cells displayed a strong G0/G1 arrest in response to gefitinib, while no significant cell-cycle alterations were seen in the slow-growing STC-1. Vice versa, the proapoptotic effects of gefitinib, as determined by caspase-3 activation and DNA fragmentation, were most pronounced in the slow-growing STC-1 cells. Using cDNA microarrays, we found extensive changes in the expression of genes involved in the regulation of apoptosis and cell cycle after incubation with gefitinib. Among them, an upregulation of the growth arrest and DNA damage-inducible gene GADD153 was observed. Phosphorylation of ERK1/2, which inhibits GADD153 expression, was reduced in a time-dependent manner. However, no gefitinib-induced activation of the GADD153-inducing p38 mitogen-activated protein kinase was detected. Our data demonstrate that the inhibition of EGFR-TK by gefitinib induces growth inhibition, apoptosis and cell-cycle arrest in NE gastrointestinal tumour cells. Thus, EGFR-TK inhibition appears to be a promising novel approach for the treatment of NE tumour disease.

Keywords: NE tumours; apoptosis; cell-cycle; EGFR; TK
Gefitinib (4-(3-chloro-4-fluorooxilino)-7-methoxy-6-(3-morpholino proposition)quinazoline (ZD1839)), a specific EGFR-TK inhibitor, is currently in clinical testing for various tumour entities (Baselga et al, 2002; Herbst, 2002). Gefitinib is a low molecular weight (MW: 447), synthetic anilinoquinazoline. The orally available and reversibly acting drug is highly specific for EGFR-TK, exhibiting almost no activity against other TKs and several serine/threonine kinases (Woodburn et al, 1998; Giardiello and Tortora, 2001). Antineoplastic properties of gefitinib have been demonstrated in a wide range of human cancers, including prostate, breast, ovarian, colon, epidermoid and lung cancer cells (Giardiello et al, 2000; Callmane et al, 2000; Sirotnak et al, 2000; Chan et al, 2001; Di Lorenzo et al, 2002; Sewell et al, 2002)

EGFR-TK inhibition has not yet been evaluated in the antineoplastic treatment of NE tumours. Hence, in the present study, we examined the antineoplastic potency of the selective EGFR-TK inhibitor gefitinib in a set of NE gastrointestinal tumour cell lines with different growth characteristics. We focused on gefitinib-induced growth inhibition, its induction of apoptosis and regulation of the cell-cycle in NE gastrointestinal tumour cells.

MATERIAL AND METHODS

Cell lines

Human pancreatic carcinoma BON cells (Ahnert-Hilger et al, 1996; Lemmer et al, 2002) were maintained in a 1 : 1 mixture of DMEM, and F12K medium containing 10% FCS (Biochrom, 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. The murine intestinal NE tumour cell line STC-1 (Höpfner et al, 2002), which was a gift from Dr D Hanahan (University of California, San Francisco, CA, USA), was cultured in DMEM supplemented with 15% horse serum (Biochrom), 2.5% FCS (Biochrom) and 1% l-glutamine. All cell lines were kept at 37°C in a humidified atmosphere (5% CO₂).

Drugs

Gefitinib was a kind gift from AstraZeneca, Great Britain. Nonradiolabelled meta-ionobenzylguanidine (MIBG) was kindly provided by Amersharm Buchler (Braunschweig, Germany). Stock solutions were prepared in DMSO and stored at -20°C. The drugs were diluted in fresh media before each experiment. In all experiments, the final DMSO concentration was <0.5%. To evaluate the effects of gefitinib and/or meta-ionobenzylguanidine, cells were incubated with either control medium or a medium containing rising concentrations of the respective drug or drug combination. Media were changed daily to ensure constant drug concentrations in the incubation medium.

Reverse transcriptase – polymerase chain reaction (RT – PCR)

The total RNA was extracted from cultured cell lines with RNAclean, following the recommendation of the manufacturer (Hybaid, London, UK). Reverse transcription and PCR reactions were carried out as described elsewhere in detail (Glassmeier et al, 1998). To eliminate any possible contamination with genomic DNA, RNAs were treated with 1 U DNase I ( Gibco, Karlsruhe, Germany) per pg RNA for 15 min at room temperature. The possible contamination with genomic DNA was excluded by control experiments omitting the reverse transcriptase. Purified RNA was reverse transcribed into cDNA using oligo-dT-primers and the SuperScript Preamplification-Kit (Gibco). PCR reactions were carried out in a total volume of 50 µl containing 400 ng of each primer, 200 µM of each dNTP (Pharmacia, Uppsala, Sweden), 50 mM KCl, 1.5 mM MgCl₂, 10 mM tris(hydroxymethyl)-amino methane (Tris) and 1 U Taq-Polymerase (Pharmacia). PCR was performed in a Peltier thermocycler (PTC-200, MJ Research, Watertown, MA, USA) with primers and conditions as indicated in Table 1.

cDNA array

For determination of gefitinib-induced differential gene expression, human CM insulinoma cells were treated with gefitinib (10 µM) for 48 h. Untreated cells served as controls. Isolation of the total RNA of treated and untreated cells was performed as described above. Polyadenylated (polyA⁺) mRNAs were enriched using magnetic Dynabeads, according to the instructions of the supplier (Dynal, Oslo, Norway). The quality of total and polyA⁺ RNA was controlled by agarose gel electrophoresis. Labelled first-strand cDNA probes were prepared from the polyA⁺ RNAs of both gefitinib-treated and control samples. Each sample was hybridised to an individual membrane of identically spotted 205 apoptosis- and cell-cycle-related genes (Human Apoptosis Array; Clontech, Palo Alto, CA, USA). A complete list of the cDNAs and controls as well as their accession numbers is available on the web (http://atlasinfo.clontech.com/genelists/huApop.xls). After washing, according to the manufacturer’s instructions, the membranes were exposed to an X-ray film for quantification. Alteration in the expression of a respective gene is given as fold increase/or decrease compared with the signal of the untreated control (Höpfner et al, 2002).

Table 1. Primer sequences and PCR conditions for the detection of mRNA expression of the indicated genes in NE gastrointestinal tumour cells

| Genes | Primers (5’–3’) | Position in the mRNA (bp) | Product size (bp) | Denaturating temperature and time (s) | Annealing temperature and time (s) | Extension temperature and time (s) | Number of cycles |
|-------|----------------|--------------------------|------------------|--------------------------------------|----------------------------------|----------------------------------|-----------------|
| EGFR-I | F: TCCTCCAGTGCCGTAATAC | 3442–3462 | 240 | 94°C (40) | 63°C (60) | 72°C (60) | 30 |
| EGFRIII | R: TACGGTTGTGCTCGCTTTC | 3682–3663 | 226 | 94°C (40) | 63°C (60) | 72°C (60) | 30 |
| IGR-R | F: GGCCTTGGAGGAAAAGAAG | 255–274 | 255 | 94°C (40) | 63°C (60) | 72°C (60) | 30 |
| β-actin | R: TCTGCGCAAGTGGTGTGTTTG | 1237–1258 | 822 | 94°C (40) | 63°C (60) | 72°C (60) | 30 |

*EGFRvIII does not possess an mRNA-sequence distinct from the EGFR-I sequence, but Exons 2 – 7 are missing. Using the indicated primers, EGFRvIII is characterised by a product of 226 bp size, while the wild-type receptor is recognised with a bp product of 1028 bp. All templates were initially denatured for 5 min at 95°C and the amplification was extended at a final extension temperature of 72°C for 7 min.*
Experimental Therapeutics

1768

mm² ratio of genes in the treated samples, whereas downregulation

antibody (5 glycerol 10%, SDS 1%, Tris-HCl 25 mM; running buffer: Tris 25 mM, glycine
electrophoresis (30 min, the lysates were subjected to SDS/polyacrylamide gel

polyclonal IGF-
cubated for 1 h at room temperature with the primary rabbit

with lysis buffer (sodium dodecyl sulphate (SDS) 0.1%, sodium
deoxycholic acid 0.5%, Nonidet P-40 1%, phenylmethylsulphonyl
fluoride (PMSF) 0.1 mM, aprotinin 1 μg ml⁻¹, pepstatin A 1 μg ml⁻¹). The protein content of the lysate was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). The cell lysate was mixed with gel-loading buffer (Tris-HCl 62.5 mM,
glycerol 10%, SDS 1%, β-mercaptoethanol 2.5%). After boiling

for 5 min, the lysates were subjected to SDS/polyacrylamide gel
electrophoresis (30 μg of protein per lane; gel: polyacrylamide 10%,
SDS 0.1%, Tris-HCl 25 mM; running buffer: Tris 25 mM, glycine
50 mM, 0.1% SDS). After electrophoresis, gels were equilibrated

with transfer buffer (Tris-HCl 25 mM, glycine 50 mM, 20% methanol). Proteins were transferred onto nitrocellulose mem-
brates by electrolotting (BioRad, Munich, Germany). Blots were

blocked in 1.5% bovine serum albumine (BSA), and then
incubated at 4°C overnight with the total p38 mitogen-activated
protein kinase (MAPK) and phospho-p38 MAPK or the total
extracellular signal-regulated kinase 1/2 (ERK1/2) and phospho-
ERK1/2 antibodies, respectively (1: 500, Santa Cruz Biotechnology,
CA, USA). After washing with phosphate-buffered NaCl solution
(PBS) containing 0.1% Tween and incubation with horseradish
peroxidase-coupled secondary antibody (1:10 000, Amersham,
Uppsala, Sweden) at room temperature for 1 h, the blot was

washed extensively and developed using enhanced chemilumines-
cent detection (Amersham, Uppsala, Sweden). Blots were exposed to
Hyperfilm ECL film (Amersham, Uppsala, Sweden) for 1–5 min.

Western blotting

Whole-cell extracts were prepared by harvesting and lysing cells
with lysis buffer (sodium dodecyl sulphate (SDS) 0.1%, sodium
deoxycholic acid 0.5%, Nonidet P-40 1%, phenylmethylsulphonyl
fluoride (PMSF) 0.1 mM, aprotinin 1 μg ml⁻¹, pepstatin A 1 μg ml⁻¹). The protein content of the lysate was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). The cell lysate was mixed with gel-loading buffer (Tris-HCl 62.5 mM,
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cent detection (Amersham, Uppsala, Sweden). Blots were exposed to
Hyperfilm ECL film (Amersham, Uppsala, Sweden) for 1–5 min.

Immunofluorescence labelling

Cells were trypsinised, washed twice with PBS and immunostained,
as described previously (Sutter et al, 2002). Then samples were
fixed and permeabilised using the Fix & Perm cell permeabilisation
kit (Caltag, Laboratories, Hamburg, Germany). Cells were incu-
bated for 1 h at room temperature with the primary rabbit
polyclonal IGF-β1 receptor antibody (5 μg ml⁻¹, # sc-713, Santa
Cruz) or with rabbit polyclonal EGF receptor antibody (5 μg ml⁻¹,
# sc-03, Santa Cruz), both mapping at the respective C-terminus of the
protein. Negative controls were performed by omitting the primary antibody. Cells were washed twice with PBS and then
incubated with secondary FITC-labelled polyclonal anti-rabbit Ig
antibody (5 μg ml⁻¹, BD Pharmingen, Heidelberg, Germany) for
1 h at room temperature. Fluorescence was detected by flow
cytometry on a FACS Calibur (Becton Dickinson, Heidelberg,
Germany) and analysed using CellQuest software.

Measurement of growth inhibition

Changes in the cell number of BON, CM and STC-1 cells were

determined by crystal violet staining after 96 h of incubation with
rising concentrations of gefitinib (0–50 μM). Measurements were
performed as described (Höpfner et al, 2001). Cells were washed
with PBS and fixed with 1% glutaraldehyde. After another washing

step, cells were stained with 0.1% crystal violet. The unbound dye
was removed by washing. Crystal violet that had absorbed onto the

cells was solubilised with 0.2% Triton X-100. Then light extinction
was analysed at 570 nm using an ELISA reader.

Caspase-3 activity assay

Preparation of cell lysates and determination of caspase-3 activity
was performed as described previously (Maaser et al, 2002). The
activity of caspase-3 was calculated from the cleavage of the
fluorogenic substrate DEVD-AMC (Calbiochem-Novabiochem,
Bad Soden, Germany). Cell lysates were incubated with substrate
solution (caspase-3 substrate AC-DEVAD-AMC 20 μg ml⁻¹, HEPES
20 mM, glycerol 10%, DTT 2 mM, pH 7.5) for 1 h at 37°C, and the
cleavage of DEVD-AMC was measured fluorometrically with a
VersaFluor fluorometer (excitation: 360 nm emission: 460 nm)
from Biorad, Munich, Germany.

DNA fragmentation

DNA fragmentation was determined by performing Cell Death
Detection ELISA (Roche) as described previously (Höpfner et al, 2003). Briefly, after 48 h of incubation, cells were lysed with
incubation buffer. The cytoplasmic fractions were diluted to contain
2.5 × 10⁶ cell equivalents per ml, and the presence of mono- and oligonucleosomes was assayed using antibodies
directed against DNA and histones. DNA fragments were detected by
a peroxidase system with colour development read at 405 nm.

Cell-cycle analysis

Cell-cycle analysis was performed by the method of Vindelov and
Christensen, as described previously (Maaser et al, 2001). Cells
were trypsinised, washed and the nuclei were isolated using the
CycleTest PLUS DNA Reagent Kit (Becton Dickinson, Heidelberg,
Germany). DNA was stained with propidium iodide according to the
manufacturers’ instructions. The DNA content of the nuclei
was detected by flow cytometry and analysed using CellFit software
(Becton Dickinson).

Statistical analysis

The antineoplastic effects of the various substances and vehicles
were compared by the unpaired, two-tailed Mann–Whitney
U-test. The unpaired Student’s t-test was used for cell-cycle analysis. P-
values were considered to be significant at <0.05. If not stated
otherwise, all functional experiments were performed in quad-
ruplicate.

RESULTS

Expression of EGFR and IGFR in NE gastrointestinal
tumour cells

The mRNA expression of EGFR receptors (EGFR) and the insulin-
like growth factor receptor β-1 (IGFR-β1) was investigated in
human BON and in human CM cells. The mRNAs specific for
EGFR and IGFR-β1 were detected in both the cell lines (Figure 1A,
B). To evaluate protein expression of both EGFR and IGFR-β1,
cells were stained with specific antibodies and analysed by flow
cytometry. Protein expression of EGFR and IGFR-β1 was detected in
both the cell lines (Figure 1C, D). By contrast, no expression of
the EGFR mutation, EGFRvIII, often observed in non-NE cancer
types, was detected in the human NE tumour models used (Figure
1A, B). Labelling murine STC-1 cells with specific antibodies for
EGFR and IGFR-β1 also confirmed the expression of both growth
factor receptors in this model of NE gut tumour cells (Figure 1D).
Growth-inhibitory effects of gefitinib

Changes in the cell number caused by EGFR-TK inhibition were studied by performing crystal violet assays. Gefitinib (0–50 μM) time- and dose-dependently inhibited the growth of all cell lines investigated (Figure 2A–C). After 96 h of incubation, a decrease of almost 100% was observed. However, the IC50 values of gefitinib, determined after 48 h, differed between the three cell lines. While in fast-growing CM cells (doubling time: 21 ± 1 h) the IC50 value of gefitinib amounted to 4.7 ± 0.6 μM, it was 16.8 ± 0.4 μM in the moderate-growing BON cells (doubling time: 34 ± 4 h), but as high as 31.5 ± 2.5 μM in the slow-growing STC-1 cells (doubling time: 54 ± 6 h) (Figure 2D).

Figure 1 mRNA and protein expression of EGFR and IGFR in neuroendocrine tumour cells. (A, B) mRNA expression of EGFRvIII (lane 1), EGFR-1 (lane 2) and IGFRβ-1 (lane 3) was evaluated in CM (A) and BON tumour cells (B). β-Actin was used as positive control (lane 4 in A and B). 100 bp DNA ladder. (C–E) Flow cytometric analysis of the expression of EGFR and IGFRβ-1 proteins in CM cells (C), BON cells (D) and STC-1 cells (E). Black lines: cells stained with specific polyclonal antibodies against either EGFR or IGFRβ-1; grey lines: negative controls.

Figure 2 Gefitinib-induced growth inhibition. Gefitinib caused a time- and dose-dependent growth inhibition as measured by crystal violet staining. The IC50 value was 4.7 ± 0.6 μM in fast-growing CM cells (A), 16.8 ± 0.4 μM in moderate-growing BON cells (B), and 31.5 ± 2.5 μM in slow-growing STC-1 cells (C). (D) Correlation between the doubling time of NE gastrointestinal tumour cells and their sensitivity to gefitinib treatment. Data are given as the percentage of untreated controls (means ± s.e.m. of four to five independent experiments). * Statistical significance (P < 0.05).
Recently, we showed that nonradiolabelled MIBG specifically inhibited the growth of norepinephrine transporter (NET)-positive NE gastrointestinal tumour cells with an IC\textsubscript{50} value of 7.8 µM (Höpfner et al, 2002). Thus, it was intriguing to evaluate the possible synergistic antiproliferative effects of the combination of gefitinib with MIBG. Incubating NET-positive STC-1 cells for 3 days with combinations of sub-IC\textsubscript{50} concentrations of MIBG (5 µM) and gefitinib (10 µM) resulted in an overadditive growth-inhibitory effect. While each drug alone decreased the growth of STC-1 cells by either 23% (10 µM gefitinib) or 28% (5 µM MIBG), the combination of both drugs led to a synergistic antiproliferative effect of more than 90% growth inhibition (data not shown).

**Gefitinib and cell-cycle regulation**

To test whether cell-cycle-arresting effects contributed to the antiproliferative potency of gefitinib in NE gastrointestinal tumour cells, we performed flow cytometric cell-cycle analysis. Challenging CM and BON cells with rising concentrations of gefitinib (0–10 µM in CM, and 0–50 µM in BON cells) for 48 h dose-dependently arrested CM and BON cells in the G1/G0 phase of the cell cycle, thereby decreasing the proportion of cells in the S phase and G2/M phase (Figure 3A, B). Interestingly, gefitinib did not affect the cell cycle of STC-1 cells, even at the highest concentration of 50 µM (Figure 3C).

**Proapoptotic effects of gefitinib**

To check whether the induction of programmed cell death contributed to the antineoplastic effects of gefitinib, we investigated gefitinib-induced caspase-3 activation and DNA fragmentation in CM, BON and STC-1 cells.

Caspase-3 is a key enzyme in the apoptotic signalling pathway. In all the three cell lines, gefitinib induced a dose-dependent increase in caspase-3 activity (Figure 4). The extent of caspase-3 activation differed between the three cell lines. Interestingly, caspase-3 activation was most pronounced in the slow-growing STC-1 cells (746 ± 122% increase), which on the other hand had not displayed cell-cycle alterations in response to gefitinib treatment. This suggests that the antiproliferative effect of gefitinib in STC-1 cells is mainly caused by an induction of apoptosis. On the other hand, fast-growing CM cells, which exhibited the most pronounced G0/G1-arrest upon gefitinib treatment, showed the lowest, albeit still distinctive, caspase-3 activation of the three NE cell lines studied.

The fragmentation of DNA into mono- and oligonucleosomes is a hallmark of apoptosis. Gefitinib dose-dependently induced DNA fragmentation in NE gastrointestinal tumour cells (Figure 5). After 48 h of incubation, an increase in DNA fragmentation of up to approx. 380% of the control values was observed. The results on DNA fragmentation confirm the notion that gefitinib potently induces apoptosis in NE gastrointestinal tumour cells. The extent of DNA fragmentation corresponded to the findings of caspase-3 activation in the respective cell line. Thus, strongest DNA fragmentation was seen in STC-1 cells, while CM cells showed less pronounced DNA fragmentation after incubation with gefitinib.

Since the antiproliferative effect of MIBG on NET-positive STC-1 cells was mainly caused by an induction of apoptosis (Höpfner et al, 2002), we wondered whether the overadditive antiproliferative effect of MIBG plus gefitinib was due to a synergistic induction of apoptosis. After 48 h of incubation, DNA fragmentation induced by either drug amounted to 116% (10 µM gefitinib) or 105% (1 µM MIBG), respectively. Combined incubation of STC-1 cells with MIBG (1 µM) and gefitinib (10 µM) revealed an overadditive activation of DNA-fragmentation of 141%.

**Gefitinib-induced differential gene expression**

The present work demonstrates that gefitinib induces both apoptosis and cell-cycle arrest in NE gastrointestinal tumour cells. To study the involved cell-cycle and apoptosis genes at the transcriptional level, we performed cDNA microarray analysis of gefitinib-treated CM cells. For microarray experiments, we chose a microarray experiment for each cell line are shown. The difference of the proportion of cells in a particular phase of the cell cycle was significant for 7.5–10 µM gefitinib in CM cells and for 10–50 µM gefitinib in BON cells. * Statistical significance (P < 0.05).

**Figure 3** Induction of cell-cycle arrest by gefitinib. After 48 h of incubation with gefitinib, CM cells (A) and BON cells (B) dose-dependently accumulated in the G0/G1-phase of the cell cycle. Accordingly, the proportion of cells in the S and G2/M phases decreased in either cell line. In contrast, no significant changes of cell-cycle phases were observed in STC-1 cells (C). Means ± s.e.m. of four independent experiments for each cell line are shown. The difference of the proportion of cells in a particular phase of the cell cycle vs control was significant for 7.5–10 µM gefitinib in CM cells and for 10–50 µM gefitinib in BON cells. * Statistical significance (P < 0.05).

Among the genes known to be involved in the apoptosis signalling cascade, the expressions of caspase 4, PDCD 2, BCL-2-antagonist of cell death (BAD) and the bcl-2 family protein harakiri were overexpressed in gefitinib-treated cells. GADD 153, which plays an important role both in apoptosis and G0/G1 arrest, was also upregulated. Among
DISCUSSION

EGFR signalling impacts on many aspects of tumour biology. The activation of EGFR has been shown to enhance tumour growth, invasion and spreading, and to inhibit apoptosis (Moghal and Sternberg, 1999; Wells, 2000). In addition, the expression of EGFR in tumours has been correlated with disease progression, poor survival, poor response to therapy (Brabender et al, 2000) and the development of resistance to cytotoxic agents (Wosikowski et al, 1997; Meyers et al, 1998). Thus, specific inhibition of EGFR and its intrinsic TK activity by different strategies (e.g. monoclonal antibodies, TK-inhibitors) has become a rationale for innovative cancer treatment (Giardiello et al, 2000; Cullinan et al, 2000; Sirotznak et al, 2000; Overholser et al, 2000; Chan et al, 2001; Di Lorenzo et al, 2002; Sewell et al, 2002; Mendelsohn, 2003), – especially since the EGFR receptor system is not only involved in tumour growth but also plays a significant role in tumour invasion, angiogenesis and adhesion (Woodburn, 1999; Ritter and Arteaga, 2003).

In the present study, we provide evidence that NE gastrointestinal tumour cells express both EGFR and the EGFR transactivating IGFR-1 (Gilmore et al, 2002). Moreover, gefitinib, a specific EGFR-TK inhibitor, was found to inhibit potently the growth of NE gastrointestinal tumour cells by inducing cell-cycle arrest and/or apoptosis. The inhibition of EGFR-TK by gefitinib led to a time- and dose-dependent growth inhibition by almost 100%. The effects were observed under foetal calf- and/or horse serum-containing conditions, that is, in the presence of growth factors like EGF, IGF and TGF-β. Under these in vivo-like serum conditions, gefitinib was very potent, and thus qualifies as a promising novel drug to be tested clinically in metastatic NE gastrointestinal tumour disease.

The growth pattern of NE gastrointestinal tumours exhibits an astonishingly wide spectrum ranging from very slow- to moderate-growing types and finally to fast-growing, very aggressive types of tumours (Oberg, 1994). In view of this particular background, it

the genes being downregulated, the cell-cycle regulating kinases ERK1, CDC-like kinase 3, cyclin-dependent kinase 10 and the DNA-replication factor proliferating cell nuclear antigen (PCNA) were found.

Gefitinib-mediated phosphorylation of ERK1/2 and p38 MAPK

To shed light on the signalling pathways influenced by EGFR-TK inhibition, we investigated time-dependent alterations of the phosphorylation of ERK1/2 and p38 MAPK, which are members of the MAPK family known to be involved in EGFR signalling in non-NE tumours. Incubating CM cells for 0–48 h with 10 μM gefitinib revealed a time-dependent decrease in the phosphorylation of mitogenic ERK1/2 (Figure 6A). In contrast, no significant alteration in the phosphorylation of the stress-activated p38 MAPK was observed (Figure 6B).
Table 2  Transcripts differentially regulated in CM cells in response to gefitinib

| GenBank ID | Gene name | Function | Ratio |
|------------|-----------|----------|-------|
| U28014     | caspase 4 | Caspase  | 4.13  |
| M63167     | roc protein kinase alpha | Apoptosis-associated protein | 3.13  |
| S78085     | programmed cell death 2 | Death kinase | 3.27  |
| U66879     | BCL2-antagonist of cell death (BAD) | Apoptosis-associated protein | 2.81  |
| Y00285     | insulin-like growth factor 2 receptor | bcl-2 family protein | 2.55  |
| U91985     | DNA fragmentation factor alpha | Growth factor receptor | 2.52  |
| M354110    | insulin-like growth-factor binding protein 2 | DNA fragmentation protein | 2.41  |
| AF02385    | programmed cell death 10 | Growth factor-binding protein | 2.40  |
| X97978     | cyclin D1 | Death receptor | 2.22  |
| M81934     | cell division cycle 25B | cyclin | 2.22  |
| M32315     | tumour necrosis factor receptor superfamily, member 1B | cyclin | 2.19  |
| U66469     | cell growth regulatory with ring finger domain | cell cycle protein | 2.19  |
| U76376     | harakiri, BCL2-interacting protein | bcl-2 family protein | 2.18  |
| H125753    | cyclin B1 | cyclin | 1.81  |
| X66363     | PCTAIRE protein kinase 1 | cell cycle-related protein kinase | 1.76  |
| U25265     | mitogen-activated protein kinase 5 | intracellular kinase network member | 1.76  |
| S40706     | GADD153 | apoptosis-related protein | 1.72  |
| U78798     | TNF receptor-associated factor 6 | death receptor-associated protein | 1.72  |
| X85134     | Retinoblastoma-binding protein 5 | cell cycle protein | 1.71  |
| L25080     | ras homologue gene family, member A | oncogene | 1.70  |
| M13228     | v-ras myelocytomatosis viral-related oncogene | oncogene | 1.68  |
| M32394     | tumour necrosis factor receptor superfamily, member 1A | death receptor | 1.67  |
| M15796     | proliferating cell nuclear antigen | DNA replication | 0.65  |
| D21090     | RAD23 homologue B | DNA damage signalling protein | 0.65  |
| S72008     | cell division cycle 10 | cell cycle protein | 0.63  |
| U39657     | mitogen-activated protein kinase 6 | intracellular kinase network member | 0.63  |
| X66362     | PCTAIRE protein kinase 3 | cdc2-related protein kinase | 0.61  |
| U1791      | cyclin H | cyclin | 0.58  |
| X60188     | extracellular signal-regulated kinase 1 (ERK1) | cell cycle-regulating kinase | 0.55  |
| L05624     | mitogen-activated protein kinase 1 | intracellular kinase network member | 0.55  |
| U75285     | survivin | apoptosis-associated protein | 0.54  |
| X79389     | glutathione S-transferase theta 1 | xenobiotic transporter | 0.54  |
| U78876     | mitogen-activated protein kinase 3 | intracellular kinase network member | 0.50  |
| U21092     | TNF receptor-associated factor 3 | death receptor-associated protein | 0.47  |
| L07414     | tumour necrosis factor superfamily, member 5 | death receptor ligand | 0.42  |
| M73812     | cyclin E1 | cyclin | 0.26  |

was important to check whether gefitinib was similarly capable of inhibiting the proliferation of NE tumours with different growth characteristics and to study the involved signalling pathways. Therefore, we chose three models of NE gastrointestinal tumour disease, each characterised by a different growth pattern and different origin: first, human insulinoma CM cells, which have a doubling time of 21 h and represent ‘fast-growing’ NE tumour cells. Second, human pancreatic carcinoid BON cells with a doubling time of 34 h, which represent ‘moderate-growing’ NE tumour cells. Third, ‘slow-growing’ NE tumour cells of the gut (STC-1 cells) with a doubling time of 54 h. Each cell line was cultured under its optimal growth conditions, which resulted in different serum concentrations among the three cell lines. Nevertheless, the different serum concentrations were not responsible for the different antineoplastic efficacies of gefitinib observed in the three cell lines (data not shown). Although gefitinib effectively inhibited tumour growth in each model, differences concerning dose–response relationships and involved signalling pathways became apparent. In fast-growing CM cells that displayed the highest sensitivity to gefitinib (IC50 of 4.7 ± 0.6 μM), the antineoplastic effect was based on both a moderate induction of apoptosis and a strong G1/G0 phase arrest of the cell-cycle. In moderate-growing BON cells also an induction of apoptosis as well as an arrest in the G1/G0 phase of the cell-cycle were observed. However, the concentrations needed to induce the antineoplastic effects were significantly higher (IC50 of 6.8 ± 0.4 μM), and the apoptotic response was more pronounced than in CM cells. On the other hand, in slow-growing STC-1 cells displaying the lowest
sensitivity to gefitinib (IC₅₀ 31.5 ± 2.5 μM), the antineoplastic effect was based on a strong induction of apoptosis, while no significant cell-cycle alterations were observed.

The shift from a more cell-cycle arrest related action in fast-growing NE tumour cells to an (almost) exclusively apoptosis-related effect in slow-growing NE tumour cells may be important to be taken into account when considering novel combination therapies. In this respect, we were interested in the antiproliferative synergism between gefitinib and the norepinephrine derivative MIBG. Nonradiolabelled MIBG has recently been suggested as an innovative drug for the treatment of NE gastrointestinal tumours expressing plasma membraneous norepinephrine transporters (NET) (Taal et al., 1996; Höpfner et al., 2002). Since the covalent pleural mesothelioma (Jenne et al., 2002) and neuroendocrine gastrointestinal tumour cells (OÈ berg, 1994). Thus, EGFR-TK inhibition might as well be a worthwhile strategy to pursue for the control of the desmoplastic reaction and fibrosis of carcinoid diseases.

Induction of apoptosis by gefitinib has been reported previously (Ciardiello et al., 2000; Huang et al., 2002; Janmaat et al., 2002). However, the underlying mechanisms are not yet well understood (Mendelsohn, 2002). In this paper, we report on the activation of the proapoptotic enzyme caspase-3, which preceded the fragmentation of nuclear DNA into oligonucleosomes. This indicates that in NE tumour cells, gefitinib-mediated apoptosis is caspase-3 dependent. To support our functional data, we additionally performed cancer-specific cDNA arrays, spotted with genes related to proliferation, apoptosis and cell cycle. Treatment with gefitinib resulted in an upregulation of proapoptotic and a suppression of antiapoptotic genes of NE gastrointestinal tumour cells (Table 2). The changes in the expression pattern of the several apoptosis-related genes were consistent with both our functional data, and reports showing that suppression of antiapoptotic and overexpression of proapoptotic proteins caused programmed cell death (Zamzami et al., 1996). Besides an overexpression of the cell cycle and apoptosis relevant GADD153 gene (see below), a strong upregulation of caspase-4, which has been implicated to play an important role in the execution of apoptosis (Kamada et al., 1997), was observed. We also observed a strong induction of proapoptotic BAD at the transcriptional level, suggesting a potential involvement of this protein in gefitinib-induced apoptosis. As suggested by others, a dephosphorylation of Akt/PKB by gefitinib may be responsible for the activation of proapoptotic BAD (Albanell et al., 2001). However, further investigations will have to clarify the exact role of BAD in gefitinib-induced apoptosis of NE tumour cells.

Gefitinib’s mode of action is known to include the suppression of EGFR-TK-induced activation of MAP kinases, such as ERK1/2 (Albanell et al., 2001; Mendelsohn, 2002). Confirming this notion, gefitinib did cause a time-dependent dephosphorylation and transcriptional downregulation of ERK1/2 in NE tumour cells. In addition, we observed an upregulation of the growth arrest and DNA-damage inducible gene, GADD153, which is involved in G1/G0 arrest (Wang and Ron, 1996; Oh-Hashi et al., 2001). The expression of GADD153 is known to be regulated by both ERK1/2 and p38 MAPK. While p38 MAPK stimulates the expression of GADD153, ERK1/2 inhibits the expression (Kultz et al., 1998; Oh-Hashi et al., 2001). Interestingly, we did not observe any significant effect of gefitinib on p38 MAPK activity in NE tumour cells. This suggests that the upregulation of GADD153 by gefitinib is mediated by a downregulation of ERK1/2, but not by increased p38 MAPK activity.

Although gefitinib’s antiproliferative effect results from the specific inhibition of EGFR-sensitive TK activity, gefitinib’s antineoplastic potency not simply reflects the number of EGFRs expressed. While some studies have reported a positive correlation between EGFR expression and gefitinib’s antineoplastic action (Meye et al., 2001; Normanno et al., 2002), others have found no such relationship (Ciardiello et al., 2000; Sirotnak et al., 2000). One possible explanation for a missing correlation between EGFR expression and gefitinib’s antiproliferative activity may be the presence of constitutively active EGFR mutations, such as EGFRvIII (Moscatello et al., 1995; Wikstrand et al., 1995; Nishikawa et al., 1994). However, in neither NE gastrointestinal tumour model used in this study, an expression of EGFRvIII mutation could be detected and hence could not contribute to the differing effects of gefitinib. Another reason for a missing positive correlation could be EGFR transactivation by other growth factor receptors (Arteaga, 2002). In this respect, Gilmore et al. (2002) recently demonstrated a transactivation of EGFR-TK by the IGF. Gefitinib suppressed the EGFR transactivation by IGF, and thereby induced apoptosis in breast cancer cells. EGFR transactivation may also be important in NE gastrointestinal tumours in which IGF is commonly expressed and has been implicated in growth control (Nilsson et al., 1993; Wulbrand et al., 2000).

To conclude, our study provides evidence that the EGFR-TK inhibitor gefitinib induces both cell-cycle arrest and apoptosis in NE gastrointestinal tumour cells. Thus, gefitinib represents a promising novel drug to be tested in patients with metastatic NE gastrointestinal tumour disease.

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