Anti-tumour activity of afatinib, an irreversible ErbB family blocker, in human pancreatic tumour cells

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BACKGROUND: The combination of the reversible epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) erlotinib with gemcitabine obtained FDA approval for treating patients with pancreatic cancer. However, duration of response is often limited and there is currently no reliable predictive marker.

METHODS: We determined the sensitivity of a panel of human pancreatic tumour cell lines to treatment with afatinib, erlotinib, monoclonal antibody (mAb) ICR62, and gemcitabine, using the Sulforhodamine B colorimetric assay. The effect of these agents on cell signalling and cell-cycle distribution was determined by western blot and flow cytometry, respectively.

RESULTS: At 200 nM, ICR62 had no effect on growth of these tumour cells with the exception of BxPC-3 cells. BxPC-3 cells were also sensitive to treatment with afatinib and erlotinib with respective IC₅₀ values of 11 and 1200 nM. Compared with erlotinib, afatinib was also more effective in inhibiting the growth of the other human pancreatic tumour cell lines and in blocking the EGF-induced phosphorylation of tyrosine, EGFR, MAPK, and AKT. When tested in BxPC-3 xenografts, afatinib induced significant delay in tumour growth.

CONCLUSION: The superiority of afatinib in this study encourages further investigation on the therapeutic potential of afatinib as a single agent or in combination with gemcitabine in pancreatic cancer.

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Pancreatic cancer remains one of the most aggressive types of human cancer. Despite major advances in imaging technologies, surgery, chemotherapy and radiotherapy in the last few decades, survival rates for patients with pancreatic cancer are extremely poor with a mean 5-year survival rate of <10% (Klapman and Malafa, 2008). Worldwide, pancreatic cancer is responsible for >200,000 deaths each year (Parkin et al, 2005; Ferlay et al, 2010). In the United States alone, there were an estimated 43 140 new cases of pancreatic cancer and 36 800 deaths in 2010 (Jemal et al, 2010). One major contributing factor to mortality is the lack of specific marker(s) for the early detection of pancreatic cancer. Indeed, ~80% of pancreatic cancer cases are diagnosed at an advanced stage of the disease and these patients are intrinsically resistant to treatment with radiation and chemotherapy (Li and Sai, 2009).

Since the early 1980s, aberrant expression and activation of epidermal growth factor receptor (EGFR) (ErbB1/HER1), which is the prototype member of the class-I growth factor receptor tyrosine kinase family (also called ErbB/HER family), has been reported in a wide range of human malignancies and in some cases it has been associated with poor prognosis (Modjtahedi and Dean, 1994; Nicholson et al, 2001; Normanno et al, 2006; Zhang et al, 2007). The ErbB/HER family consists of three additional members namely ErbB2 (neu/HER2), ErbB3 (HER3) and ErbB4 (HER4) (Normanno et al, 2006). The binding of a HER ligand to its respective receptor leads to conformational changes in the receptor’s extracellular domain which allow the formation of homo- and/or heterodimers between different members of the ErbB family. Dimerisation results in auto- and transphosphorylation of specific tyrosine residues in the cytoplasmic region of the receptors, which in turn leads to the activation of numerous downstream mediator molecules. These downstream proteins mediate the activation of several signalling pathways the most significant of which are the PI3K/Akt and the RAS/RAF/MAPK pathways. These have an important role in the regulation of cellular processes such as cell proliferation, differentiation and survival (Olayioye et al, 2000; Yarden, 2001; Normanno et al, 2006). The association between aberrant activation of ErbB receptor family and a poor prognosis in a number of studies has led to the strategic development and approval of several ErbB targeted agents for the treatment of several human malignancies. These include anti-HER monoclonal antibodies (mAbs) like trastuzumab, cetuximab and panitumumab and small molecule HER tyrosine kinase inhibitors (TKIs) such as erlotinib, gefitinib and lapatinib (Zhang et al, 2007). Since the introduction of gemcitabine only the combination with erlotinib has been tested and approved for the treatment of patients with advanced pancreatic cancer (Kelley and Ko, 2008). This combination led to a marginal but statistically significant improvement in overall survival (6.24 months in combination vs 5.91 months with gemcitabine alone) and an increase in 1-year survival rate (23% with the combination vs 17% with gemcitabine alone; Moore et al,
Despite the clinical benefit for a small fraction of treated patients, the majority of pancreatic cancer patients simply do not respond to treatment with erlotinib or acquire resistance following a few rounds of drug administration. Therefore, it is imperative not only to develop novel and more effective therapeutic agents for the treatment of pancreatic cancer, but also to identify those factors that are responsible for the poor response or development of resistance to the different types of therapies.

We reported previously the development of a large panel of anti-EGFR mAbs, from which ICR62 was found to inhibit the binding of ligands to the EGFR and the subsequent ligand-induced EGFR tyrosine phosphorylation and activation of downstream cell signalling pathways (e.g., MAPK and PI3-K phosphorylation; Modjtahedi et al., 1993, 2003; Cunningham, 2006). In addition, mAB ICR62 has been shown to inhibit the growth of the EGFR overexpressing cell lines both in vitro and in vivo (Modjtahedi et al., 1993, 1996; Cunningham, 2006). Since small molecule TKIs and mAbs target two different domains of the receptor (extracellular and intracellular domains, respectively), a combination of these two strategies may be more effective than treatment with single agents. Indeed, in some studies, dual targeting of the EGFR with a combination of the two approaches (i.e., cetuximab with erlotinib or gefitinib) was shown to be superior to treatment with a single agent (Huang et al., 2004; Matar, 2004; Regales et al., 2009).

Unlike the reversible EGFR TKIs (gefitinib or erlotinib), afatinib (BIBW 2992) is an anilino-quinazoline derivative that was designed to covalently bind and irreversibly inhibit the ErbB family members EGFR, HER2 and HER4 (Solca, 2007; Minkovskaya and Berezov, 2008; Perera et al., 2009; Nam et al., 2011). Afatinib was shown to have superior growth inhibitory efficacy compared with erlotinib in preclinical lung cancer models and in particular against lung cancer cells which display the EGFR (T790M) mutation and which are resistant to treatment with erlotinib or gefitinib (Li et al., 2008). In this study, we investigated the sensitivity of seven human pancreatic tumour cell lines to treatment with mAb ICR62, afatinib, erlotinib and gemcitabine used alone or in combinations. In addition, we investigated whether there was an association between the expression level of ErbB family members (EGFR, HER-2, HER-3 and HER-4) and response to treatment with the anti-HER agents as well as the effect of these agents on the cell-cycle distribution of pancreatic cancer cell lines.

MATERIALS AND METHODS

Tumour cell lines

A panel of seven human pancreatic cancer cell lines was used in this study including BxPC-3, PT-45, MiaPaCa-2, PANC-1, AsPC-1, Capan-1 and FA6. AsPC-1 and Capan-1 cell lines were kindly provided by Dr Charlotte Edling (Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry). Other pancreatic tumour cell lines (PANC-1, MiaPaCa2 and BxPC-3) were purchased from European Collection of Cell Cultures (ECACC, Porton Down, UK). The EGFR overexpressing (MDA-MB468), HER2 overexpressing (SKBR3) and MCF-7 human breast tumour cell lines were used as controls in this study (Cunningham, 2006). All cell lines were cultured routinely at 37 °C in a humidified atmosphere (5% CO2). MiaPaCa2 and PANC-1 were cultured in Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich, Gillingham, UK) supplemented with 10% Fetal Bovine Serum (FBS) (heat inactivated; PAA Laboratories, Yeovil, UK) and antibiotics penicillin (50 units per ml), streptomycin (0.05 mg ml^-1) and neomycin (0.1 mg ml^-1) (Sigma-Aldrich). BxPC-3, PT-45, AsPC-1, Capan-1 and FA6 were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS, antibiotics penicillin, streptomycin and neomycin and 2 mM Glutamine (Sigma-Aldrich).

Antibodies and other reagents

mAb ICR62 (IgG2b) was raised against the external domain of the EGFR on the breast cancer cell line MDA-MB468 as described previously (Modjtahedi et al., 1993). The primary mouse antibodies used in this study, HM50.67A and HM43.16B, were raised against the external domain of the HER-2 and EGFR, respectively (Cunningham, 2006). Mouse mAbs MAB3481 (anti-HER-3) and MAB11311 (anti-HER-4) were purchased from R&D Systems (Abingdon, UK). Secondary FITC-conjugated rabbit anti-mouse mAb STAR9B was obtained from AbD Serotec (Kidlington, UK). Erlotinib was kindly provided by OSI Pharmaceuticals (Farmingdale, NY, USA). Gemcitabine was acquired from Healthcare at Home (Burton on Trent, UK) and the irreversible ErbB family blocker afatinib was developed by Boehringer Ingelheim (Vienna, Austria).

Cell surface expression of HER family members

The cell surface expression of all HER family members (EGFR, HER-2, HER-3 and HER-4) was assessed by flow cytometry as described previously (Cunningham, 2006). Briefly, ~1 × 10⁶ cells were suspended in 1 ml of growth medium supplemented with 2% FBS and incubated for 1 h by rotation at 4 °C, with medium alone or medium containing 10 μg ml⁻¹ of mouse primary antibodies. Cancer cells were then washed three times by centrifugation at 1200 r.p.m. (264 g) for 5 min, resuspended in 0.5 ml of FACS Flow buffer (Becton Dickinson Ltd, Oxford, UK). A minimum of 10,000 events were recorded by excitation with an argon laser at 488 nm using the FL-1 detector (525 nm) of a BD FACS Calibur flow cytometer (Becton Dickinson Ltd). Mean fluorescence Intensity values were calculated using the CellQuest Pro software (Becton Dickinson Ltd) and compared with those of negative controls.

Growth inhibition studies

The effect of the various agents on the growth of human cancer cell lines was investigated using the Sulforhodamine B (SRB; Sigma-Aldrich) colorimetric assay. Briefly, tumour cells were seeded at a density of 5 × 10⁴ cells per well in 100 μl of growth medium (RPMI-1640 or DMEM according to the cell line) supplemented with 2% FBS in a 96-well plate. After 4 h incubation at 37 °C (in a humidified atmosphere, in 5% CO₂), 100 μl aliquots of doubling dilutions of the agents were added to triplicate wells. Cells were incubated at 37 °C (3–5 days depending on the cell line) until the control cells (no treatment) became confluent. Tumour cells were then fixed with 10% trichloracetic acid (Fisher Scientific, Loughborough, UK) for 1 h, washed five times with tap water, air dried and stained with 1% sulforhodamine B (Sigma-Aldrich) colorimetric assay. Briefly, washed cells were treated with 100 μl of solution (1% acetic acid, air dried and stained with 0.4% SRB in 1% acetic acid for 1 h. Following that, cells were washed with 1% acetic acid, air dried and the SRB stain was solubilised with 10 mM Tris-base (100 μl per well; Fisher Scientific). The absorbance of each well was measured at 565 nm using an Epoch plate reader (Biotek, Potton, UK). For the determination of the initial number of cells (before treatment), an extra plate was set up and processed similarly after 4 h incubation at 37 °C without the inhibitors. Growth as a percentage of control was determined from the formula:

\[
\% \text{Growth} = \frac{B - A}{C - A} \times 100
\]

where \( A \) = Absorbance at 565 nm (A565 nm) before treatment, \( B \) = A565 nm after treatment with inhibitors and \( C \) = A565 nm after incubation in medium alone. The 50% inhibitory concentration of cell growth (IC50) was calculated by non-linear least squares curve
Determination of combination index
The growth inhibitory effect of the agents under investigation was also assessed when used in combination. Interactions between the different agents were assessed, using the combination index (CI) as described by Chou and Talalay (1984). For each combination, the two drugs were mixed at their 4 × IC50, followed by eight doubling dilutions. Combination index < 0.9 indicates a synergistic effect while CI between 0.90 and 1.10 denotes an additive effect. Combination index > 1.1 indicates antagonistic effects. Data analysis was performed using the CalcuSyn software (Biosoft, Cambridge, UK).

Cell-cycle distribution analysis
The effect of HER inhibitors and gemcitabine on the cell-cycle distribution of the cancer cell lines was investigated using flow cytometry. Briefly, ~2.5 × 10^6 cells were seeded to 25 cm^2 flasks containing 10 ml of 2% FBS growth medium and the inhibitors at different concentrations or control medium. Once the cells containing only medium were almost confluent, treated cells were harvested and pooled together with the supernatant. Cancer cells were washed three times with cold PBS by centrifugation at 1200 r.p.m. (264 g) for 5 min. The final cell pellet was resuspended in 200 µl of cold PBS, fixed by the addition of 70% ethanol overnight at 4 °C. Tumour cells were then collected by centrifugation (264 g for 5 min) washed once with PBS and incubated with PI/RNAse mix (500 µl per 10^6 cells) (Becton Dickinson Ltd) for 35 min at room temperature. A minimum of 10 000 events were recorded by excitation with an argon laser at 488 nm using the FL-3 detector (620 nm) of a BD FACS Calibur Flow cytometer (Becton Dickinson Ltd) and analysed using the CellQuest Pro software (Becton Dickinson Ltd).

Western blot analysis
Cancer cells were grown to near-confluency in 6-well culture plates containing 5 ml of 10% FBS RPMI growth medium. Cells were washed once with 5 ml of RPMI/0.5% FBS and incubated in 5 ml of RPMI/0.5% FBS containing no inhibitor, afatinib (400 nM), erlotinib (400 nM), ICR62 (400 nM) or gemcitabine (100 nM) for 24 h at 37 °C. Following incubation with the inhibitors, cells were stimulated with 20 nM of EGF (Austral Biologicals, San Ramon, CA, USA) for 15 min. Following incubation with the inhibitors, cells were stimulated with 20 nM of EGF (Austral Biologicals, San Ramon, CA, USA) for 15 min and washed once with 5 ml of PBS. Cancer cells were lysed using 400 µl of Lysis buffer (Invitrogen, Paisley, UK) containing a protease inhibitor cocktail (Sigma-Aldrich) and cell lysates were heated at 90 °C for 4 min. Protein samples (20 µg) were separated on 4–12% Bis-Tris gels (Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membranes using the XCell II Surelock Mini-Cell System kit (Invitrogen) and probed with antibodies to HER family members (EGFR, HER-2, HER-3 and HER-4) in human pancreatic cancer cell lines (Table 1). The WesternBlot analysis was performed using the Cell Signalling Technology rabbit antibodies from Sigma-Aldrich. Rabbit antibodies against Akt, MAPK and phospho-MAPK (Thr202/Tyr204) were purchased from Cell Signalling while anti-phospho EGFR (Tyr1173) and anti-phospho Akt (S473) rabbit antibodies were obtained from Biosource (Paisley, UK). The specific signals were detected using the WesternBreeze chemiluminescence kit (alkaline phosphatase-conjugated secondary antibody) (Invitrogen).

In vivo xenograft experiments
Five- to six-week-old female athymic BomTac:NMRI-Foxn1nu mice were maintained under specific pathogen-free conditions. All experiments complied with the Declaration of Helsinki and European Policy Legislations (FELASA and GV-SOLAS) on the Care and Use of Laboratory Animals. After acclimatisation mice were inoculated subcutaneously with 1 × 10^6 BxPC-3 cells (in 100 µl matrigel) into the right flank of the animal. After tumours reached an average volume of 50–100 mm^3, the mice were randomised and treated orally daily with 15 mg kg⁻¹ afatinib or vehicle control on the basis of individual weights. Tumours were measured three times a week with calipers, and tumour volumes were calculated by the formula $\pi/6 \times \text{length} \times \text{width}^2$. Afatinib was formulated in 1.8% HP-beta-CD (Hydroxypropyl-β-cyclo-dextrin, Sigma-Aldrich), 5% of a 10% acetic acid stock and aqueous Natrosol (0.5%), and administered by intragastral gavage. The administration volume was 10 ml kg⁻¹ body weight.

Statistical analysis
Linear regression analysis was used for the assessment of the relationship between the HER family receptors expression and response to treatment with afatinib. The unpaired two-tailed Student’s t-test was used for comparing mean values between two groups. Data are presented as mean ± s.d. $P<0.05$ was considered statistically significant.

RESULTS
Cell surface expression of HER family members
We determined the expression levels of HER family of receptors in seven different pancreatic cancer cell lines using flow cytometry. All pancreatic tumour cell lines were found to be positive for both EGFR and HER-2 (Table 1). Compared with the MFI value of 415 in the positive-control EGFR overexpressing cell line MDA-MB468, the MFI values for the EGFR in this panel of human pancreatic tumour cell lines were lower and ranged from 34.54 (FA6) to 29.25 (MiaPaCa-2) (Table 1). Most of the human pancreatic tumour cell lines were found to be HER-4 negative while expressing extremely low or undetectable levels of HER-3 (Table 1).

Growth response of human pancreatic tumour cells to treatment with anti-EGFR mAb ICR62, erlotinib, afatinib and gemcitabine
Of the seven human pancreatic tumour cell lines examined, BxPC-3 cells were the most sensitive cell line to treatment with afatinib and EGFR inhibitor mAb ICR62. All other pancreatic cancer cell lines were much lower and showed a synergistic response to treatment with afatinib and EGFR- and HER-4-targeting agents (Table 1). The growth inhibitory effects of the agents under investigation were retested in the presence and absence of the HER inhibitors to confirm the contribution of HERs in the growth control of the cells. All agents were able to inhibit the growth of all cell lines, suggesting that the HERs are involved in the growth control of these cell lines.

Table 1 Expression (MFI) of HER family members (EGFR, HER-2, HER-3 and HER-4) in human pancreatic cancer cell lines

| Cell line       | Control MFI | EGFR MFI | HER-2 MFI | HER-3 MFI | HER-4 MFI |
|-----------------|-------------|----------|-----------|-----------|-----------|
| Capan-1         | 4.2 ± 0.7   | 138.5 ± 15.2| 28.9 ± 3.1| 7.4 ± 0.7 | 4.9 ± 0.4 |
| PT-45           | 2.9 ± 0.6   | 1847.7 ± 21.1| 21 ± 1.9 | 61 ± 0.4  | 3.9 ± 0.3 |
| PANC-1          | 3.0 ± 0.2   | 119.7 ± 7.6| 13.6 ± 0.6| 3.4 ± 0.1 | 4.1 ± 0.2 |
| BxPC-3          | 8.1 ± 1.2   | 980.0 ± 12.7| 294.9 ± 3.3| 15.8 ± 1.5| 9.8 ± 0.5 |
| MiaPaCa-2       | 3.7 ± 0.3   | 395 ± 4.3  | 329 ± 3.8 | 4.8 ± 0.3 | 4.1 ± 0.3 |
| AsPc-1          | 3.6 ± 0.7   | 974.7 ± 12.8| 19 ± 2   | 59 ± 0.5  | 4.1 ± 0.3 |
| FA6             | 5.9 ± 0.4   | 40.4 ± 8.2 | 14.5 ± 1.9| 9.8 ± 1.4 | 5.5 ± 1.3 |
| SKBR3 (breast)  | 4.3 ± 0.2   | N/A       | 831.3 ± 74.6| 21.2 ± 8.7| N/A       |
| MDA-MB468 (breast) | 3.8 ± 0.3  | 415 ± 3.4 | N/A       | N/A       | N/A       |

Abbreviation: EGFR = epithelial growth factor receptor; Breast cancer cell lines SKBR3, MDA-MB468 and MCF-7 were used as positive controls for HER-2/HER-3, EGFR and HER-4 receptors, respectively.
with an IC50 value of 11 nM. The growth of other human pancreatic tumour cells was also inhibited by afatinib with IC50 values ranging from 367 nM (AsPc-1) to 1.37 μM (FA6) (Table 2; Figure 1B). In addition, BxPC-3 cells were the most sensitive to treatment with erlotinib with an IC50 value of 1.26 μM followed by AsPc-1 with an IC50 value of 5.8 μM (Table 2; Figure 1C). The mAb ICR62 has previously been shown to completely inhibit the growth of EGFR overexpressing tumour cell lines HNS and DFP in vitro in the low nanomolar range. In these experiments, ICR62 did not have any effect on the growth of the human pancreatic tumour cell lines tested at 200 nM (Figure 1D; Cunningham, 2006). The only exception was BxPC-3 cells, which were growth inhibited by 13%, however, with no statistical significance (P = 0.061, Figure 1D). Gemcitabine inhibited the growth of all human pancreatic tumour cell lines with IC50 values ranging from 5 nM (FA6) to 105 nM (Capan-1) (Figure 1A; Table 2). Morphology of BxPC-3 cells following the treatment with growth inhibitory concentrations of erlotinib, afatinib and gemcitabine is presented in Figure 2. Next, we investigated the interactions between the different agents when used in combination. Median effect analysis showed that a combination of afatinib or erlotinib with gemcitabine led to a synergistic effect (CI < 0.9) in the majority of cell lines tested (Table 2). We found no enhancement of growth inhibition following treatment with a combination of ICR62 with afatinib or gemcitabine or erlotinib in these in vitro models (data not shown).

**Correlation of HER family member expression and sensitivity to ErbB inhibitors**

Linear regression analysis was carried out in order to determine whether sensitivity to treatment with ErbB inhibitors was dependent on the expression level of the HER receptors. There was no correlation between the expression of EGFR (R = 0.010, \( P = 0.982 \)) or HER-2 (R = 0.458, \( P = 0.301 \)) and the IC50 values for afatinib nor any association between EGFR expression levels and response to treatment with erlotinib (R = 0.153, \( P = 0.743 \)) or other members of HER family.

**Cell-cycle distribution analyses**

We examined the changes in cell-cycle distribution of the pancreatic cancer cells of various origins after treatment with afatinib and gemcitabine. Treatment with gemcitabine increased the proportion of pancreatic tumour cells in the subG1 phase (apoptotic/dead cells) of the cell cycle and this was accompanied by a significant increase in the percentage of cells in S phase consistent with inhibition of DNA synthesis (Table 3). For example, treatment of BxPC-3 cells with gemcitabine increased the proportion of cells in the subG1 phase from 9% to 36% and in the S phase from 22% to 46%, respectively (Table 3). In contrast, treatment of human pancreatic cells with afatinib increased the proportion of cells in subG1 phase of the cell cycle, accompanied by a decrease in the percentage of cells in G0/G1 phase of the cell cycle in the majority of cell lines investigated. Erlotinib had a similar effect to afatinib on the cell-cycle distribution of the two cell lines examined (BxPC-3 and AsPc-1) (Table 3).

**Effect of anti-EGFR mAb ICR62, erlotinib, afatinib and gemcitabine treatment on EGF-induced tyrosine phosphorylation of the proteins in BxPC-3 cells**

In contrast to gemcitabine, all three inhibitors blocked the EGF-induced tyrosine phosphorylation of EGFR and the phosphorylation of the tyrosine residue at position 1173 of the EGFR in BxPC3 cells. However, of the three inhibitors, afatinib was the only agent that successfully blocked the EGF-induced phosphorylation of both MAPK (ERK 1/2) and Akt (Figure 2B).

**Xenograft experiments in mice**

*In vivo*, afatinib showed potent anti-tumour activity in the BxPC-3 human pancreatic xenograft model. Daily administration of 15 mg kg\(^{-1}\) afatinib as a single agent to mice carrying established tumours did not induce long-lasting tumour regressions but significantly delayed tumour growth with a T/C value of 18% and a tumour growth inhibition (TGI) value of 89% as assessed at the end of the experiment (Figure 3).

**DISCUSSION**

Currently, among the several ErbB-targeting inhibitors developed, only the reversible EGFR TKI erlotinib has been approved in combination with gemcitabine for the treatment of patients with pancreatic cancer (Mendelsohn and Baselga, 2006; Ciardiello and Tortora, 2008; Modjtahedi and Essapen, 2009). While erlotinib improves survival, the duration of response is often limited and the majority of patients acquire resistance following a few rounds of treatment. Unlike first-generation reversible EGFR TKIs such as erlotinib and gefitinib, afatinib covalently binds and irreversibly inhibits the tyrosine kinase activity of all ErbB family members and is therefore expected to block both partners in the ErbB receptor dimer. This should result in a more effective signalling blockade and greater anti-tumour efficacy when compared with agents targeting EGFR alone. In this study, for the first time, we investigated the growth response of a panel of human pancreatic tumour cells to treatment with afatinib, ICR62, erlotinib and gemcitabine when used alone or in combination. We also

| Cell line | Erlotinib IC50 (μM) | Afatinib IC50 (μM) | Gemcitabine IC50 (μM) | Afatinib+gemcitabine combination index (effect) | Erlotinib+gemcitabine combination index (effect) |
|-----------|---------------------|-------------------|-----------------------|--------------------------------------------|--------------------------------------------|
| BxPC-3    | 1.26 μM             | 11 μM             | 7.4 μM                | 0.31 (Synergism)                           | 0.46 (Synergism)                           |
| AsPc-1    | 5.8 μM              | 367 μM            | 87.3 μM               | 0.62 (Synergism)                           | 0.69 (Synergism)                           |
| FA6       | > 20 μM             | 1.37 μM           | 5.23 μM               | 0.85 (Slight synergism)                    | N/A                                       |
| PANC-1    | > 20 μM             | 966 μM            | 21.4 μM               | 0.73 (Moderate synergism)                  | 0.66 (Synergism)                           |
| Capan-1   | > 20 μM             | 781 μM            | 104.9 μM              | 0.88 (Slight synergism)                    | N/A                                       |
| MiaPaCa-2 | > 20 μM             | 709 μM            | 6.4 μM                | 1.25 (Moderate antagonism)                 | 1.30 (Moderate antagonism)                 |
| PT-45     | > 20 μM             | 1.02 μM           | 14.3 μM               | 1.40 (Moderate antagonism)                 | 0.54 (Synergism)                           |

Abbreviations: N/A = not available; IC50 = 50% inhibitory concentration; SRB = Sulforhodamine B. Interpretation of the results was based on the proposed descriptions for presenting the degrees of antagonism or synergism by CalcuSyn software.

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investigated whether there was any association between the expression of HER family members and response to treatment with ErbB inhibitors.

We reported previously that, at concentrations > 3.2 nM, mAb ICR62 inhibits completely the growth of the EGFR overexpressing cell lines HN5 and DiFi in vitro (Cunningham, 2006). In the present experiments, at a maximum concentration of 200 nM, mAb ICR62 had no significant effect on the growth of any of the human pancreatic tumour cell lines investigated (Figure 1D). This may be due to the lower expression of EGFR in these pancreatic cell lines compared with the EGFR expression in HN5 or DiFi cells (Modjtabadi et al, 1993; Cunningham, 2006). Another possible explanation for the lack of response to treatment with mAb ICR62 could be the presence of autocrine loops. Previous studies have shown that EGFR ligands may compete with mAbs for binding to the receptor and reduce their anti-proliferative activity (Castillo, 2004). In another study, Buchsbaum and colleagues investigated the effect of anti-EGFR mAb erbitux on the proliferation of BxPC-3 and MiaPaCa-2 cells. They found that while exposure to erbitux for 96 h resulted in a 17% reduction in proliferation of BxPC-3 cells, it did not have any inhibitory effect on MiaPaCa-2 cells (Buchsbaum et al, 2002). Of all cell lines examined in our study, mAb ICR62 only inhibited the growth of BxPC-3 cells by 13%, but this was not statistically significant (P = 0.061, Figure 1D) and blocked the EGFR-induced tyrosine phosphorylation of EGFR in these cells (Figure 2B).

Unlike mAb ICR62 which targets the extracellular ligand binding domain of the EGFR, afatinib is an irreversible inhibitor of the ErbB family (Solca, 2007; Berezov, 2008; Minkovsky and Berezov, 2008; Nam et al, 2011). Afatinib was found to inhibit the growth of all seven human pancreatic cancer cell lines with IC50 values ranging from 11 nM (BxPC3) to 1.37 μM (FA6) (Figure 1B; Table 2). However, the majority of cell lines were found to be insensitive to treatment with erlotinib with only BxPC-3 and AsPc-1 cell lines responding to treatment with this agent. The IC50 values were 1.26 and 5.8 μM for BxPC-3 and AsPc-1 cells, respectively (Table 2). In agreement with our findings in a previous study, Buck et al (2006) investigated the sensitivity of three pancreatic tumour cell lines, BxPC-3, MiaPaCa-2 and PANC-1 to treatment with erlotinib and of these only BxPC-3 was marginally sensitive to treatment with erlotinib with an IC50 value of 1.3 μM. In another study, erlotinib was found to inhibit the growth of BxPC-3 and Capan-1 cells with IC50 values of 7.6 and > 20 μM, respectively (Cai et al, 2010). This comparative data analysis suggests that afatinib inhibits more potently the proliferation of pancreatic cancer cells in vitro. In addition, of all agents investigated, only afatinib inhibited the EGF-induced phosphorylation of MAPK (ERK 1/2) and Akt in BxPC-3 cells. Several studies have evaluated the anti-tumour activity of lapatinib, which is a reversible dual EGFR/HER-2 TKI. In one study, Komoto et al (2010) found that lapatinib, at the maximum concentration of 30 μM, inhibited the growth of Capan-1, Capan-2, PANC-1 and MiaPaCa-2 by < 8%. Recently, in a recent phase II study 125 patients with metastatic pancreatic cancer were treated with 1500 mg of Lapatinib per day and 1g m for 3 weeks until the disease progression. However, no therapeutic benefit was reported for such combination (Safran et al, 2011).

In this study, we found no correlation between the expression level of EGFR (R = 0.010, P = 0.982) or HER-2 (R = 0.458, P = 0.301) and response to treatment with afatinib. Similarly, Komoto et al (2010) found that there was no statistically significant association between EGFR or HER-2 expression and the in vivo anti-tumour effect of the dual EGFR/HER-2 TKI lapatinib. These findings suggest that ErbB receptor expression levels are not of major relevance and may not be used as predictive biomarkers to identify responding models in vitro. Other factors such as the mutational status of ErbB family receptors or downstream

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**Figure 1** Effect of doubling dilutions of gemcitabine (A), afatinib (B) or erlotinib (C) on the growth of human pancreatic cancer cells. Tumour cells were grown in growth medium (2% FBS) with the inhibitors or medium alone until control cells (only medium) were confluent. Cancer cell proliferation was calculated as a percentage of control cell growth, as described in the Materials and Methods. Each point represents the mean ± s.d. (D) The effect of gemcitabine at 100 nM, ICR62 at 200 nM and erlotinib or afatinib at 1.5 μM in pancreatic cancer cell lines (as percentage of control growth) is shown (columns, mean of triplicate values; bars, ± s.d.).

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molecules like K-RAS may be implicated in the sensitivity to ErbB inhibition. For example, in glioblastoma and non-small-cell lung carcinoma (NSCLC) patients the presence of EGFRvIII or T790M EGFR was found to be associated with resistance to treatment with gefitinib and erlotinib, respectively (Learn et al., 2004; Kobayashi et al., 2005; Pao et al., 2005). In addition, K-Ras mutations, which lead to the activation of the K-RAS protein in an EGFR-independent manner, have been associated with resistance to treatment with EGFR targeting mAbs in NSCLC and metastatic colorectal cancer patients (Linardou et al., 2008). While EGFR gene mutation is a rare phenomenon in pancreatic cancer, the presence of K-Ras mutations is a very common event and occurs in 75–95% pancreatic cancer patients (Hruban et al., 1993; Berrozpe et al., 1994; Laghi et al., 2002; Immervoll et al., 2006). It is noteworthy that of all the cell lines investigated in this study, BxPC-3 is the only one which carries a wild-type K-Ras gene which could explain its greater sensitivity to afatinib (IC50 = 11 nM) and erlotinib (IC50 = 1.26 μM) treatment compared with the rest of the tested cell lines (Table 2; Sipos et al., 2003). Further studies aimed at replacing wild-type K-Ras with mutant K-Ras in isogenic models.

Figure 2  (A) Morphology of BxPC-3 cells following growth inhibitory concentrations of erlotinib, afatinib and gemcitabine (μM) compared with treatment with medium alone (original magnification ×20). (B) Effect of afatinib, erlotinib and ICR62 on EGF-induced phosphorylation of tyrosine, EGFR, MAPK and Akt in BxPC-3 cells. BxPC-3 cells were cultured to near-confluency in growth medium containing 10% FBS, then treated in 0.1% FBS medium containing 400 nM of TKI, mAb ICR62 (400 nM) or gemcitabine (100 nM) for 24 h at 37°C. Following incubation with the inhibitors, cells were stimulated with 20 nM of EGF for 15 min. Then, treated cells were lysed, protein samples were separated by SDS–PAGE, transferred onto PVDF membranes, and probed with antibodies specific for the molecule of interest.
In conclusion, our results show that afatinib is effective at inhibiting the growth of human pancreatic tumour cells and that it potentiates the effect of gemcitabine in the majority of cell lines examined. Taken together, our findings encourage future investigation on the therapeutic potential of afatinib, as a single agent or in combination with gemcitabine in pancreatic cancer.

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