Insulin-like growth factor-I receptor signalling and acquired resistance to gefitinib (ZD1839; Iressa) in human breast and prostate cancer cells

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Abstract

De novo and acquired resistance to the anti-tumour drug gefitinib (ZD1839; Iressa), a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) has been reported. We have determined whether signalling through the IGF-I receptor (IGF-1R) pathway plays a role in the gefitinib-acquired resistance phenotype. Continuous exposure of EGFR-positive MCF-7-derived tamoxifen resistant breast cancer cells (TAM-R) to 1 \( \mu M \) gefitinib resulted in a sustained growth inhibition (90%) for 4 months before the surviving cells resumed proliferation. A stable gefitinib-resistant subline (TAM/TKI-R) was established after a further 2 months and this showed no detectable basal phosphorylated EGFR activity. Compared with the parental TAM-R cells, the TAM/TKI-R cells demonstrated (a) elevated levels of activated IGF-1R, AKT and protein kinase C (PKC)\( \delta \), (b) an increased sensitivity to growth inhibition by the IGF-1R TKI AG1024 and (c) an increased migratory capacity that was reduced by AG1024 treatment. Similarly, the EGFR-positive androgen-independent human prostate cancer cell line DU145 was also continuously challenged with 1 \( \mu M \) gefitinib and, although substantial growth inhibition (60%) was seen initially, a gefitinib-resistant variant (DU145/TKI-R) developed after 3 months. Like their breast cancer counterparts, the DU145/TKI-R cells showed increases in the levels of components of the IGF-1R signalling pathway and an elevated sensitivity to growth inhibition by AG1024 compared with the parent DU145 cell line. Additionally, DU145/TKI-R cell migration was also decreased by this inhibitor. We have therefore concluded that in breast and prostate cancer cells acquired resistance to gefitinib is associated with increased signalling via the IGF-1R pathway, which also plays a role in the invasive capacity of the gefitinib-resistant phenotype.

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Introduction

The epidermal growth factor receptor (EGFR) or erbB1 is a proto-oncogene, which encodes a 170 kDa transmembrane protein (Gullick 2001). The cytoplasmic portion of the receptor possesses intrinsic tyrosine kinase (TK) activity, which is activated following ligand binding to the extracellular domain of the receptor (Gullick 2001).

Ligand-induced EGFR activation initiates a signal transduction cascade, which mediates cellular processes such as proliferation, migration and differentiation. Overexpression of EGFR is linked with the pathogenesis of many human solid tumour types and has been correlated with poor prognosis and implicated in disease progression, playing a key role in promoting tumour invasion and metastasis (Salomon et al. 1995, Nicholson et al. 2001). In...
addition to EGFR over-expression, many solid tumours also demonstrate elevated expression of its cognate ligands, EGF and transforming growth factor-\( \alpha \) (TGF-\( \alpha \)) (Di Marco et al. 1989, Salomon et al. 1995), suggesting that the receptor may be activated in an autocrine manner. In breast cancer, for example, EGFR over-expression has been associated with oestrogen receptor-negative disease and hence with poor prognosis (Nicholson et al. 1994, 2001) and in vitro studies have also implicated EGFR in the mediation of acquired resistance to anti-oestrogen therapy (van Aghthoven et al. 1992, Miller et al. 1994, McClelland et al. 2001, Knowlden et al. 2003). Similarly, EGFR signalling has been linked to the progression of androgen-responsive prostate cancer to androgen-independent/hormone refractory tumours (Djakiew 2000). Elevated expression of both EGFR and its ligands have been described in prostate tumours (Djakiew 2000) and in vitro studies have indicated that the growth of the androgen-independent prostate tumour cell line DU145 is regulated by the autocrine activation of the EGFR by its ligands EGF and TGF-\( \alpha \) (Connolly & Rose 1991). Furthermore, preclinical data have suggested that the EGFR signalling pathway can activate the androgen receptor under conditions of clinical androgen deprivation (Barton et al. 2001).

Thus, the EGFR signalling pathway represents a key target for intervention in cancer therapy. Numerous strategies have been employed to specifically target EGFR, such as monoclonal, bispecific and single chain antibodies, antisense oligonucleotides and small molecule inhibitors of the EGFR TK activity (TK inhibitors (TKIs)) (Ciardiello & Tortora 2001). The inhibitors prevent EGFR signalling by directly inhibiting receptor autophosphorylation. For example, the anilooquinazoline gefitinib competitively inhibits binding of ATP to the receptor kinase domain (Wakeling et al. 2002). Gefitinib has been shown to inhibit the growth of a range of human tumour xenografts including breast, prostate, colon and lung and, furthermore, significantly potentiates the anti-tumour activity of a variety of cytotoxic agents when used in combination (Ciardiello et al. 2000, Wakeling et al. 2002). Recent phase II/III clinical studies have demonstrated that gefitinib monotherapy was well tolerated and provided anti-tumour activity in patients with advanced non-small cell lung carcinoma (NSCLC), prostate and breast cancer and also colorectal, renal and head and neck cancers (Barton et al. 2001, Cohen et al. 2002, Fukuoka et al. 2002, Goss et al. 2002, Herbst & Kies 2002, Ranson 2002). Additionally, studies are also currently evaluating the effect of gefitinib in combination with other therapies in a range of tumour types including prostate, breast, NSCLC, colorectal, head and neck, ovarian, bladder and cervical cancers (Hammond et al. 2001, Gonzalez-Larriba et al. 2002, Herbst et al. 2002, Trump et al. 2002).

Gefitinib presents much therapeutic promise for the treatment of numerous human cancer types; however, the clinical data demonstrated that not all patients responded to the inhibitor, indicating the existence of an intrinsic or de novo resistance to the drug (Barton et al. 2001, Cohen et al. 2002, Fukuoka et al. 2002, Goss et al. 2002, Herbst & Kies 2002, Ranson 2002). Furthermore, even with responders to gefitinib, it was observed that disease progression could occur within a few months of therapy (Barton et al. 2001, Cohen et al. 2002, Fukuoka et al. 2002, Goss et al. 2002, Herbst & Kies 2002, Ranson 2002) resulting from the development of acquired resistance to the TKI. The acquisition of resistance to gefitinib has also been demonstrated in vitro, with the establishment of a gefitinib-resistant PC-9 NSCLC cell line, being observed following a stepwise dose escalation of the compound over 1 year (Yamakoa et al. 2002). Furthermore, other studies have demonstrated that resistance to the inhibitor developed after several generations of treatment in prostate tumour xenografts (Geller et al. 2002). Development of resistance has also been described with other signal transduction inhibitors such as traztuzumab (Herceptin). Resistance to this human antibody directed against the extracellular domain of erbB2 (HER,neu), a related EGFR TK family member and used in the treatment of erbB2-positive metastatic breast cancer, has been reported to occur within 12 months (Baselga 2001, Slamon et al. 2001).

It is feasible that resistance to gefitinib may be acquired by the ability of the tumour cells to utilise alternative growth factor pathways in the presence of the drug. Indeed, it has recently been shown in several different cancer types that the action of EGFR and erbB-2 signal transduction inhibitors could be acutely modulated by the insulin-like growth factor-I receptor (IGF-1R) (Liu et al. 2001, Lu et al. 2001, Chakravati et al. 2002). The IGF-1R is a transmembrane TK receptor which is activated by binding of its ligands IGF-I and IGF-II and extensive studies have established that the receptor signalling plays an important role in tumorigenesis, metastatic potential of the tumour cell and neoplastic growth (Long et al. 1998, Yu & Rohan 2000, Pollak 2001). In breast cancer, over-expression and activation of IGF-1R and its downstream signalling molecules have been linked to disease progression, increased resistance to radiotherapy and poor prognosis (Rocha et al. 1997, Turner et al. 1997). Elevated levels of IGF-1R and its ligands also appear to contribute to prostate cancer pathogenesis as increased IGF-1R expression has been found in primary prostate cancers compared with benign prostate epithelium (Hellawell et al. 2002) and high levels
of plasma IGF-I are associated with increased risk of prostate cancer (Pollak 2001).

**In vitro** studies to date have only implicated the IGF-1R in the acute interference of anti-EGFR/erbB2 strategies. In this current study we have therefore aimed to approach the clinical scenario by continuously exposing EGFR-positive cell lines to gefitinib for a period of several months and, in addition, choosing two cancer types currently being assessed in clinical trials for the inhibitor, namely breast and prostate cancer. The role of IGF-1R in mediating proliferation and invasion in the subsequently generated cell variants with acquired resistance to the inhibitor has been investigated. The study was initiated by modelling resistance to the TKI using a breast cancer cell line derived from EGFR-positive tamoxifen-resistant MCF-7 variant cells (TAM-R). The TAM-R cell line was generated in our laboratory as an in vitro model to study acquired resistance to the anti-hormonal agent tamoxifen, a phenomenon which is a major clinical problem in the treatment of breast cancer (Gee et al. 2002). Briefly, hormone-responsive MCF-7 cells were continuously exposed to tamoxifen and consequently, on acquisition of tamoxifen resistance, the TAM-R cells demonstrated a marked increase in EGFR expression and a reliance on this receptor signalling for growth as shown by their good initial growth-inhibitory responses to gefitinib (Knowlden et al. 2003). The subsequent gefitinib-resistant cell population was derived by long-term exposure to the previously determined effective inhibitory dose of the compound. In order to determine whether IGF-1R signalling may provide a common mechanism of resistance to the TKI, these studies were complemented by assessing the role of this growth factor pathway in gefitinib-resistant prostate cells derived from the androgen-independent EGFR-positive DU145 human prostate cancer cell line.

Such studies provide unique data regarding the underlying signal transduction mechanisms involved in the development and progression of breast and prostate cancer during gefitinib treatment. It is hoped that they may generate novel means of extending the quality and duration of response to this class of drugs and aid the determination of optimum therapeutic regimes.

**Materials and methods**

**Routine cell culture**

The cell lines were routinely cultured in the following basal media: the MCF-7-derived TAM-R (Knowlden et al. 2003) and TAM/TKI-R breast cancer cell lines were maintained in phenol-red free RPMI containing 5% charcoal stripped fetal calf serum (FCS), penicillin–streptomycin (10 IU/ml–10 μg/ml), fungizone (2.5 μg/ml) and glutamine (4 mM), together with 0.1 μM 4-hydroxytamoxifen (4-OH-TAM) or a combination of 0.1 μM 4-OH-TAM and 1 μM gefitinib respectively both compounds were gifts from AstraZeneca (Macclesfield, UK). The androgen-independent DU145 human prostate cancer cell line (Stone et al. 1978) was grown in Dulbecco’s minimum Eagle’s medium (DMEM) supplemented with 10% FCS plus antibiotics and its gefitinib-resistant variant DU145/TKI-R was maintained in serum-free DCCM-1 (Biological Industries, Cumberland, UK) with 1 μM gefitinib. It should be noted that although maintained in DCCM-1, the DU145/TKI-R cells were initially seeded out in serum-supplemented DMEM culture medium for 24 h prior to maintenance in serum-free DCCM-1 with gefitinib. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. All tissue culture media and constituents were purchased from Gibco Europe Ltd (Paisley, Strathclyde, UK) and tissue culture plastic ware was obtained from Nunc (Roskilde, Denmark).

**Establishment of cell lines resistant to the EGFR TKI gefitinib**

The TAM-R cell cultures were washed with Dulbecco’s phosphate-buffered saline (PBS) and continuously exposed to gefitinib (1 μM) in routine culture medium which was replaced every 4 days. Initially, TAM-R cell numbers were dramatically reduced and during the following 3 months the surviving cells were passaged approximately every 14 days with a seeding ratio of 1:2. Cell proliferation slowly increased to passage every 10 days with the seeding ratio increasing 1:4 over the next 2 months. A stable growth rate was reached after a total of 6 months with routine maintenance of the TAM/TKI-R cells involving passage every 7 days with a seeding ratio of 1:10 of the confluent cell number. Similarly, a DU145 prostate cancer cell line resistant to gefitinib, described as DU145/TKI-R, was also established by chronic exposure to the inhibitor at a concentration of 1 μM in serum-free DCCM-1 medium. Following approximately 60% initial growth inhibition, the wild-type (WT) DU145 cells showed very slow but steady increases in growth, with a stable growth rate being reached after 3 months. Routine maintenance of the DU145/TKI-R cultures consisted of passage every 7 days with a seeding ratio of 1:40.

**Experimental cell culture**

Cell monolayers of each cell line were trypsinised and resuspended in the appropriate medium as detailed in the section on Routine cell culture. The cultures were seeded into 24-well plates at a density of 4 × 10⁵ cells/well for TAM-R and TAM/TKI-R cells and 5 × 10⁵ cells/well for WT DU145 and DU145/TKI-R cells. After 24 h, the cells
were washed with PBS and various experimental treatments were added as detailed below. Furthermore, all experimental media also contained 0.1 μM 4-OH-TAM, 0.1 μM 4-OH-TAM/1 μM gefitinib or 1 μM gefitinib for TAM-R, TAM/TKI-R and DU145/TKI-R cells respectively, unless stated otherwise. The experimental medium was replaced every 4 days.

**Growth curve analysis**

The effect of a variety of treatments on the proliferative capacity of the parental and gefitinib-resistant breast and prostate cell lines was determined. Treatments included (a) the effect of 1 μM gefitinib on the parental TAM-R and WT DU145 cells in basal routine medium and (b) challenge with various growth factors, i.e. including EGF, TGF-α, IGF-I, IGF-II, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and heregulin-β (all at 10 ng/ml) in phenol red-free, serum-free DCCM-1 medium, with the addition of 1 μM gefitinib for the gefitinib-resistant breast and prostate cell lines. Cell population growth was evaluated in triplicate cultures by Coulter counter analysis (Coulter, Luton, UK). Each experiment was performed in triplicate.

**Dose–response studies**

The effect of varying concentrations of several compounds on the anchorage-dependent growth of TAM-R, TAM/TKI-R, WT DU145 and DU145/TKI-R cells in their basal routine medium was assessed. Treatments included (a) gefitinib (1–10 μM) and (b) the IGF-1R inhibitor AG1024 (Calbiochem) (Parrizas et al. 1997) at a concentration of 1–10 μM. Cell growth was determined in triplicate cultures per experiment by Coulter counting on day 10 post-treatment and, additionally, each experiment was performed in triplicate.

**Immunocytochemical studies**

Each cell line was resuspended in the appropriate basal medium and seeded onto 22 mm² glass cover-slips coated with aminopropyltriethoxysilane at a density of 1×10⁵ cells/dish for TAM-R and TAM/TKI-R cells and 7.5×10⁵ cells/dish for WT DU145 and DU145/TKI-R cells. After 7 days growth, immunocytochemistry was performed as detailed below. Both positive (i.e. MCF-7 cells with 15 min IGF-I priming to promote a maximal phosphorylated IGF-1R signal) and negative controls (comprising TAM/TKI-R cells treated with the IGF-1R inhibitor AG1024, 5 μM, for 7 days) were also performed.

**Total IGF-1R**

The cover-slips were fixed by immersion in phenol formal saline (2.5% phenol in 3.7% formal saline) for 10 min at room temperature (r/t). After two washes in PBS (5 min each), the cover-slips were incubated with the rabbit polyclonal IGF-1R antibody (N-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), at a concentration of 1/125 in PBS for 24 h at r/t. This antibody recognises an epitope situated at the amino terminus of the IGF-1R α chain. Following a 3 min PBS wash and two further washes with immunostain wash solution (DPC Ltd, Llanberis, UK) (5 min each), the cover-slips were incubated with secondary anti-rabbit EnVision peroxidase-labelled polymer antibody (DAKO Ltd, Ely, Cambs, UK) for 2 h at r/t. The cover-slips were washed in PBS (1×3 min) and again with buffer solution (2×5 min) (DPC Ltd), prior to the addition of the EnVision diaminobenzidine tetrahydrochloride and hydrogen peroxide chromagen substrate (H₂O₂) for 6 min. After washing with distilled water and counterstaining with methyl green (0.5%), the cover-slips were mounted onto glass slides using a xylene-soluble mountant.

**Phosphorylated IGF-1R**

Cover-slips were fixed by sequential exposure to methanol (−20°C for 2 min), methanol/sodium vanadate (2 mM) solution (−20°C for 5 min), methanol (−20°C for 2 min) and finally acetone (−20°C for 5 min). After air-drying, the cover-slips were incubated overnight with the rabbit polyclonal anti-pY1316 IGF-1R antibody (Rubini et al. 1999) at a concentration of 1/150 in PBS for 24 h at r/t. The antibody recognises the phosphorylated tyrosine 1316 residue located in the C terminus of the IGF-1R and does not cross-react with the insulin receptor. Phosphorylated IGF-1R staining was revealed by the addition of the secondary anti-rabbit EnVision peroxidase-labelled polymer antibody and cells were counterstained as described for total IGF-1R immunocytochemistry.

**Immunostaining analysis**

Brown immunostaining at the plasma membranes and in the cytoplasm for total IGF-1R or phosphorylated IGF-1R was assessed in the cover-slips by consensus agreement of two personnel (J M W G and R I N) using a dual-viewing attachment to a light microscope (BH-2 microscope; Olympus Optical Co., Hamburg, Germany). An overall examination of immunostaining was first performed at an ocular magnification of ×10 to locate representative areas of cells for further analysis. These areas were then viewed at ×40 for more detailed tumour cell immunostaining assessment. After checking assay internal positive and negative controls for acceptable positive staining and minimal non-specific binding, cell percentages of low, moderate or high staining intensity were recorded for at least two fields (≈2000 cells)/cover-slip (with three cover-slips/treatment) so that an H-score (see equation below) or field staining index for each area were then viewed at ×40 for more detailed tumour cell immunostaining assessment. After checking assay internal positive and negative controls for acceptable positive staining and minimal non-specific binding, cell percentages of low, moderate or high staining intensity were recorded for at least two fields (≈2000 cells)/cover-slip (with three cover-slips/treatment) so that an H-score (see equation below) or field staining index for each
marker could be assigned as described previously (McClelland et al. 1991) and statistically analysed.

\[ H\text{-score} = \sum \left( \frac{\% \text{ of cells staining very weakly } \times 0.5}{} \right) + \left( \frac{\% \text{ of cells staining weakly } \times 1}{} \right) + \left( \frac{\% \text{ of cells staining moderately } \times 2}{} \right) + \left( \frac{\% \text{ of cells staining strongly } \times 3}{} \right) \]

Representative photomicroscopy was performed using an Olympus Camedia C-200 digital camera and Olympus DP software.

**Western blotting analysis**

**Experimental procedures and cell preparation**

The cell lines were seeded into 100 mm diameter dishes in basal medium, at a density of \(1 \times 10^6\) cells/cm\(^2\) for TAM-R and TAM/TKI-R and \(1 \times 10^4\) cells/cm\(^2\) for WT DU145 and DU145/TKI-R cells. After 7 days in culture, the cells were (a) harvested for analysis of basal protein expression or (b) challenged with AG1024 (1–10 \(\mu\)M) for 1–24 h or (c) exposed to IGF-I or IGF-II (10 ng/ml) for 15 min following a 24-h period in serum-free DCCM-1. Following several PBS washes, the cell monolayers were lysed at 4°C over 10 min by the addition of 50 mM Tris–HCl, pH 7.5, containing 5 mM EGTA, 150 mM NaCl, 1% Triton X100, 2 mM sodium vanadate, 200 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride, 10 \(\mu\)g/ml leupeptin, 20 \(\mu\)M phenylarsine oxide, 10 \(\mu\)g/ml aprotinin and 10 mM sodium molybdate. Lysates were clarified by centrifugation for 15 min at 13000 r.p.m. at 4°C and the protein concentration of the supernatant was determined using the BioRad DC protein assay kit (BioRad Laboratories Ltd, Hemel Hempstead, Herts, UK).

**Gel electrophoresis and immunodetection**

Protein samples (30 \(\mu\)g) from the total cell lysates were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. The membranes were blocked by the addition of 5% non-fat milk in Tris-buffered saline–Tween (10 mM Tris, pH 8, 150 mM NaCl and 0.1% Tween 20) for 1 h at r.t., followed by incubation with a variety of antibodies for 3 h at r.t. The antibodies employed were prepared in TBS-Tween containing 1% non-fat milk at the dilutions indicated below. The antibodies used were anti-EGFR (SC-03; Insight Biotechnology Ltd, Wembley, UK; 1/1000), anti-phosphorylated EGFR\(^{Y1173}\) (TCS/Upstate Biotechnology, Buckingham, Bucks, UK; 1/1000), anti-ERK1/2 (T-9102; New England Biolabs, Herts, UK; 1/1000), anti-phosphorylated ERK1/2\(^{Tyr202/Thr204}\) (A-9101; New England Biolabs; 1/4000), anti-phosphorylated protein kinase C (PKC)\(^{\delta, \theta, \varepsilon}\) (Cell Signalling, Herts, UK; 1/500), anti-phosphorylated AKT\(^{Ser473}\) (Cell Signalling; 1/1000), anti-human IGF-1sR (Sigma Chemical Co., Poole, Dorset, UK; 1/2000), anti-phosphorylated IGF-1R\(^{Y1136}\), a gift from R Baserga (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA); 1/1000), anti-phosphorylated insulin receptor (IR)IGF-1R\(^{Y1158/\varepsilon, Y1131}\) (Biosource International Inc., Nivelles, Belgium; 1/1000) and β-actin (AC-15; Sigma; 1/10 000). After three washes in TBS-Tween, the membranes were further incubated with the appropriate secondary IgG horseradish peroxidase-labelled antibody, diluted 1/10 000 in 1% milk/TBS-Tween for 1 h. Following several TBS-Tween washes, the proteins of interest were detected by West Dura, Femto or Pico chemiluminescent detection reagents (Pierce and Warriner Ltd, Chester, Cheshire, UK) as appropriate.

**RT-PCR**

For RT-PCR analysis, RNA was prepared using the RNA isolator kit (Sigma) from cultures of each cell line grown for 7 days in their basal medium as described in the section on Routine cell culture. Total RNA (1 \(\mu\)g) was reverse transcribed and the resulting cDNA was amplified using specific primer sets for heregulin (5’ GAT CAT CAC TGG TAT GCC AG and 3’ TAA ATT CAA TCC CAA GAT GC), bFGF (5’ CTG GCT ATG AAG GAA GAT G and 3’ TTA CTG GGA CAA TGG TTA C), IGF-I (5’ TGC TGT CCA GTT GGT GTG and 3’ TGG CAT GTC ACT CTT CAC TC) and IGF-II (5’ TGG GAA TCC CAA GGA AG and 3’ CTT GCC CAC GGG GTA TCT). In parallel, β-actin cDNA (5’ GGA GCA ATG ATC TTG ATC TT and 3’ CCT TCC TGG AGT CCT) was amplified in replicate samples as an internal control. Conditions for PCR amplification were as described in Knowlden et al. (2003). The resulting amplified cDNA fragments were resolved on agarose gels stained with ethidium bromide, photographed under u.v. illumination and scanned using a GS-690 Imaging Densitometer (BioRad). All densitometric scores were normalised relative to β-actin.

**Cell invasion assays**

The *in vitro* invasiveness of the parental and gefitinib-resistant cell lines was determined using a modification of the method described by Albini et al. (1987). Transwell plates equipped with 10 \(\mu\)m thick, 6.5 mm diameter polycarbonate inserts of pore size 8.0 \(\mu\)m (Costar, Cambridge, MA, USA) were precoated with 0.4 mg/ml solubilised tissue basement membrane (Matrigel; Becton Dickinson, Oxford, Oxon, UK) and allowed to dry in a sterile hood overnight. The cells were grown for 4 days in basal medium in the absence and presence
of 1 μM gefitinib. After harvesting by trypsinisation and resuspension in their basal medium plus treatment, the cells were seeded into the upper chamber at 5 × 10^4/well in a volume of 200 μl; the lower chamber was filled with 0.6ml basal medium also containing the treatment. Cells were allowed to migrate for 72 h at 37°C and 5% CO₂. At the end of the assay, non-invasive cells were removed from the upper surface of the membrane with a cotton swab. Invaded cells were fixed with 3.7% formaldehyde for 15 min at r/t prior to mounting on microscope slides using Vectorshield mounting medium (Vector Laboratories, Peterborough, UK) containing 1.5 μg/ml DAPI (Vectashield + DAPI; Molecular Probes, OR, USA). The effect of AG1024 (0–10 μM) on WT DU145 and DU145/TKI-R cell migration was investigated exactly as detailed previously. Because of the marked growth-inhibitory effect of AG1024 on both the TAM-R and TAM/TKI-R cells however, it was necessary, in order to obtain sufficient numbers of cells to accurately reproduce the invasion assay, to grow these cell lines in basal medium minus AG1024 for 4 days, prior to their reseeding onto the Matrigel, in medium containing AG1024 (0–10 μM). Cell migration was assessed after 72 h as described before.

Statistics

Overall differences between control and treatment groups were examined by means of a Kruskall–Wallis test. Direct comparisons between the parental cells and their gefitinib-resistant counterparts or between control and treatment effects were determined using a two-sided Mann–Whitney test. Significance was determined at the $P \leq 0.05$ level.

Results

Development and characterisation of the gefitinib-resistant breast cancer cell line

The TAM-R cells, originally derived from WT parental MCF-7 cells, demonstrated an increased dependency on EGFR signalling for growth as described previously (Knowlden et al. 2003). Incubation of TAM-R cells with gefitinib (1 μM) resulted in an initial dramatic reduction in cell numbers. The remaining cells demonstrated a minimal level of proliferation for approximately 3 months. After this time, slow increases in growth occurred indicating that a TAM-R cell line resistant to the growth-inhibitory properties of the TKI had developed. The growth curves presented in Fig. 1A show the initial marked growth inhibition (90%) of the TAM-R cells by gefitinib (1 μM) and the subsequent growth of the gefitinib-resistant variant (TAM/TKI-R). These dually resistant cells showed a reduced but stable growth rate compared with that of their immediate TAM-R parent after a period of 6 months (Fig. 1A). Dose–response studies showed that the TAM/TKI-R cells were insensitive to gefitinib concentrations up to 10 μM (see inset in Fig. 1A).

Assessment of the expression of EGFR and transduction molecules

Western blot analysis showed that the TAM/TKI-R cells expressed moderate levels of EGFR, compared with the parental TAM-R cells (Fig. 1B, left-hand panel). In contrast to the TAM-R cells, the EGFR was not phosphorylated (Fig. 1B, left-hand panel). Similarly, the TAM/TKI-R cells also demonstrated low levels of basal activity of the downstream EGFR target, ERK1/2, as compared with the TAM-R cells (Fig. 1B, left-hand panel). Determination of total IGF-1R protein levels indicated that the TAM/TKI-R cells expressed the receptor but at a level marginally lower than that noted in TAM-R cells (Fig. 1B, middle panel). Conversely, however, higher amounts of phosphorylated IR/IGF-1R were observed in the TAM/TKI-R cells compared with the parental cells (Fig. 1B, middle panel). Additionally, Western blot analysis of signalling elements potentially downstream of IGF-1R showed that, compared with the TAM-R parental cell line, the TAM/TKI-R cells have elevated levels of phosphorylated PKCδ and AKT (Fig. 1B, right-hand panel).

Growth responses to various mitogens and presence of growth factor ligands

Cell exposure to growth factors showed that EGF, TGF-α, IGF-I, IGF-II, bFGF and heregulin-β stimulated the growth of the TAM-R cells ($P < 0.05$), but PDGF had no effect (Fig. 2). In the presence of gefitinib, IGF-I, IGF-II, bFGF and heregulin-β increased the growth of TAM/TKI-R cells ($P = 0.004$), but no change was seen with EGF, TGF-α and, again, PDGF (Fig. 2). Moreover, direct comparison with the levels of growth stimulation shown by the TAM-R cells to IGF-I, IGF-II and heregulin-β indicated a significantly higher ($P < 0.001$) magnitude of response to these growth factors by the TAM/TKI-R cells (Fig. 2). In addition, expression studies using RT-PCR showed that of these potential growth-promoting agents, only IGF-II was produced by the TAM/TKI-R cells and at a level similar to that seen in the parental TAM-R cell line (see inset in Fig. 2).

Immunocytochemistry of IGF-1R

Immunocytochemical analysis of IGF-1R expression showed that under serum-supplemented conditions, basal total IGF-1R expression was significantly lower in the TAM/TKI-R cells (26% ($P \leq 0.001$) compared with
Figure 1 Comparative growth and protein expression characteristics of TAM-R and TAM/TKI-R cells. (A) Growth curve of TAM-R (∇), TAM-R plus 1 μM gefitinib (■) and TAM/TKI-R cells (▲) all performed in routine culture medium. Values show mean cell number ± S.D. of triplicate wells at each time-point and represent three individual experiments. The inset demonstrates the growth responses of TAM/TKI-R cells to various concentrations of gefitinib (1–10 μM) assessed on day 10 from initial treatment and represents the mean of three experiments with each point being evaluated in triplicate per experiment. (B) TAM-R and TAM/TKI-R cells were grown in routine culture medium for 7 days. Protein (30 μg) of cell lysate was electrophoresed by SDS-PAGE (7.5% gel) and immunoblotted for total and phosphorylated (phospho-) EGFR and phosphorylated ERK1/2 (left-hand panel), total IGF-1R and phosphorylated IR/IGF-1RpY1158 / pY1131 (middle panel) and phosphorylated AKT and PKCδ (right-hand panel). Densitometric analysis was performed and results were normalised to β-actin levels. The data illustrated are representative of three separate experiments.
its TAM-R parent (Fig. 3A and B and Table 1). In contrast, however, the TAM/TKI-R cell line showed significantly higher overall level of phosphorylated IGF-1R (38% increase, \(P = 0.009\)), compared with the TAM-R cells (Fig. 3C and D and Table 1). The majority of the IGF-1R immunostaining was cytoplasmic in both cell lines but both cytoplasmic and membrane-localised phosphorylated IGF-1R protein expression was significantly increased (13%, \(P \leq 0.001\) and 85%, \(P \leq 0.001\) respectively; Fig. 3C and D and Table 1) in the TAM/TKI-R cell line. An example of the negative control utilised for the immunostaining assay is also illustrated which shows that, in the TAM/TKI-R cells, the intense staining representing basal phosphorylated IGF-1R (Fig. 3E) was effectively blocked by treatment with the IGF-1R inhibitor AG1024 (Fig. 3F).

**Responses of the IGF-1R and signalling molecules to IGF stimulation**

In order to establish whether stimulation of the IGF-1R leads to increased activation of PKC\(\delta\) and AKT, molecules indirectly implicated downstream by their basal elevation in the TAM/TKI-R cells, the effect of a short-term (15 min) challenge with both IGF-I and IGF-II on the phosphorylation levels of IGF-1R, PKC\(\delta\) and AKT was undertaken. The effect of IGF stimulation on phosphorylated ERK1/2 activity was also determined. Increased phosphorylated IR/IGF-1R activity was seen in both TAM-R and TAM/TKI-R cells in response to exposure to IGF-I and IGF-II (Fig. 4). The data also indicated however that, in the TAM-R cells, the only notable change was a moderate increase in phosphorylated AKT levels after exposure to IGF-I, whereas both IGF-I and IGF-II resulted in a considerable increase in PKC\(\delta\) and AKT phosphorylation in the TAM/TKI-R cells (Fig. 4). Both ligands stimulated phosphorylated ERK1/2 activity in the two cell lines (Fig. 4).

**Effects of the IGF-1R TKI AG1024**

**Cell proliferation**

The contribution of IGF-1R to cell proliferation was determined by assessing the sensitivity of the TAM-R and
Figure 3 Immunocytochemical localisation of total and phosphorylated IGF-1R. Cells were grown in routine culture medium for 7 days and total basal IGF-1R immunostaining was determined in (A) TAM-R and (B) TAM/TKI-R cell lines. Basal levels of phosphorylated IGF-1R<sup>Y1316</sup> were also assessed in the (C) TAM-R and (D) TAM/TKI-R cells. In addition, TAM/TKI-R cells were grown in the (E) absence and (F) presence of AG1024 (5 μM) in routine culture medium for 7 days and levels of phosphorylated IGF-1R<sup>Y1316</sup> were determined.
TAM/TKI-R cell lines to AG1024, an IGF-1R TKI as described by Parrizas et al. (1997). Dose–response studies demonstrated that the proliferation of both the cell lines was reduced in a concentration-dependent manner (Fig. 5A). Compared with the TAM-R cells, however, the TAM/TKI-R cells consistently showed increased growth inhibition at each concentration of AG1024 investigated, i.e. 1–10 μM (P ≤ 0.05) (Fig. 5A).

**IGF-1R and downstream signalling**

As ERK1/2 activity appeared to be lost with chronic exposure to gefitinib, despite the increased activation of the IGF-1R, a known upstream activator of ERK1/2, the effect of IGF-1R inhibition on ERK1/2 phosphorylation was determined, in order to assess whether ERK1/2 had become uncoupled from the IGF-1R during the manipulation of the cells. It was seen that IR/IGF-1R phosphorylation was inhibited by AG1024 in both the TAM-R and TAM/TKI-R cells (Fig. 5B, see also Fig. 3E versus F). It was subsequently noted, however, that phosphorylated ERK1/2 levels were only reduced in the TAM-R cells, with ERK activation remaining unchanged in the TAM/TKI-R cells in the presence of the IGF-1R inhibitor (Fig. 5B).

The effect of the IGF-1R TKI AG1024 on the expression of phosphorylated AKT and PKCδ was assessed in the TAM/TKI-R cell line. A concentration-dependent reduction in phosphorylated AKT occurred in the presence of AG1024 (1 and 5 μM) after 1h, with this protein being barely detectable at 5 μM AG1024 (Fig. 5C). Levels of phosphorylated PKCδ were, however, seen to decrease at 5 μM AG1024 (Fig. 5C).

**Determination of invasive capacity and role of IGF-1R**

The invasive capabilities of the TAM-R and TAM/TKI-R cell lines were examined using an in vitro invasion assay. TAM-R cells invaded through the Matrigel and adhered to the underside of the porous membrane after 72 h of

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**Table 1** H-score analysis of immunocytochemistry performed on day 7 for total and phosphorylated (phospho-) IGF-1R in TAM-R and TAM/TKI-R cells. Data represent the means ± S.E.M. of the assessment of a minimum of two fields/cover-slip (with three cover-slips/treatment).

<table>
<thead>
<tr>
<th></th>
<th>TAM-R</th>
<th>TAM/TKI-R</th>
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</thead>
<tbody>
<tr>
<td><strong>Total IGF1-R</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>136±9</td>
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<td>Membrane</td>
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<td><strong>Total</strong></td>
<td>176±17</td>
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<tr>
<td><strong>Phospho-IGF1-R</strong></td>
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<td></td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>67±12</td>
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<td>Membrane</td>
<td>7±4</td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td>74±11</td>
<td>102±15</td>
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</table>

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**Figure 4** TAM/TKI-R cells show elevated activation of PKCδ and AKT after challenge with IGF-I and IGF-II, compared with the responses seen in the TAM-R cells. TAM-R and TAM/TKI-R cells were grown in routine culture medium for 7 days and, following a 24-h period in serum-free DCCM-1, were challenged with IGF-I and IGF-II (both at 10 ng/ml) for 15 min. Cell lysate was electrophoresed, immunoblotted for phosphorylated (phospho-) IR/IGF-1R Thr1151/Ser1159, PKCδ, AKT and ERK1/2 and results were normalised to β-actin levels as described in Fig. 1B. The data illustrated are representative of three separate experiments.
(A) TAM/TKI-R cells show increased sensitivity to treatment with the IGF-1R inhibitor AG1024. Growth analysis of TAM-R (solid bars) and TAM/TKI-R (spotted bars) cells grown in routine culture medium in the absence and presence of AG1024 (1–10 μM). Results are expressed as % of control values and show the means ± S.D. of triplicate wells. The data are representative of three individual experiments. *P ≤ 0.05, significant differences between the TAM-R and the TAM/TKI-R cells at each AG1024 concentration.

(B) Differential inhibition of ERK1/2 activity in the TAM-R versus TAM/TKI-R cells by the IGF-1R inhibitor AG1024. The cell lines were grown in routine culture medium for 7 days and exposed to AG1024 (10 μM) for 24 h. Cell lysate was electrophoresed, immunoblotted for phosphorylated (phospho-) IR/IGF-1R (pY1158/pY1131) and ERK1/2 and results were normalised to β-actin levels as described for Fig. 1B. Results are representative of three experiments.

(C) Treatment with AG1024 reduces the phosphorylation of AKT and PKCδ. TAM/TKI-R cells were grown in routine culture medium for 7 days and exposed to AG1024 (up to 5 μM) for 1 h. The cells were assessed for phosphorylated AKT and PKCδ and normalised to β-actin levels as described in Fig. 1B. Results are representative of three experiments.
culture (Fig. 6A). Treatment of TAM-R cells with 1 μM gefitinib for 7 days resulted in the reduction (52%, \( P < 0.05 \)) in the number of migrated cells (Fig. 6A). Interestingly, in comparison with the basal migratory capacity of the TAM-R cells and the levels shown after treatment with gefitinib, considerably greater numbers of the TAM/TKI-R cells were seen to have migrated though the Matrigel layer (120%, \( P < 0.05 \) and 320%, \( P < 0.05 \) respectively; Fig. 6A).

The role of IGF-1R in modulating the migration of the TAM-R and TAM/TKI-R cells was determined by assessing the effect of the IGF-1R TKI AG1024 on the ability of cells to migrate through the Matrigel layer. It was seen that, compared with control values, AG1024 had no effect on the migration of the TAM-R cells (\( P = 0.845 \); Fig. 6B). In contrast, it was observed that treatment with 5 and 10 μM AG1024 resulted in significant reductions of 35% and 41% respectively (\( P < 0.05 \)) in the number of migrated TAM/TKI-R cells compared with control values (Fig. 6B).

### Development of a gefitinib-resistant DU145 prostate cancer cell (DU145/TKI-R) and assessment of various components of IGF-1R signalling

It is established that WT DU145 cells utilise the EGFR autocrine regulatory loop in order to maintain growth (Connolly & Rose 1991) and anchorage-dependent growth assays showed that these cells demonstrated substantial growth inhibition (60%) following incubation with gefitinib (1 μM) (Fig. 7). The subsequent remaining cells demonstrated slow increases in growth over the following 2 months, with a stable growth rate being reached after a total of 3 months exposure to the inhibitor. This gefitinib-resistant DU145 variant (DU145/TKI-R) showed a dramatic increase in growth rate compared with that of its WT parent (Fig. 7).

Levels of components of the IGF-1R signalling pathway were subsequently determined. Expression studies using RT-PCR showed that compared with its WT parent, the DU145-TKI-R cell line demonstrated a marked increase in growth rate compared with that of its WT parent (Fig. 7).

Additional characterisation

In the DU145 cells, no significant growth increases were seen after exposure to IGF-I or IGF-II; however, both bFGF and heregulin-β increased growth by 155 ± 19% and 207 ± 20% respectively, whereas in the DU145/TKI-R cells, stimulation with IGF-I, IGF-II, bFGF and heregulin-β in the presence of 1 μM gefitinib resulted in minimal increases (less than 10%) in growth (data not shown). RT-PCR analysis indicated that the DU145/TKI-R cell line produced bFGF, but the level was reduced on comparison with the DU145 cells (see Fig. 8A). Heregulin-β mRNA was not detected in either the parental or gefitinib-resistant prostate cell lines.

### AG1024 inhibitor studies utilising the prostate cancer cells

#### Cell proliferation

Exposure to the IGF-1R inhibitor AG1024 resulted in the DU145/TKI-R cells demonstrating a marked sensitivity to the compound, showing significant decreases in growth at 5 μM (20%, \( P = 0.004 \)) and 10 μM (48%, \( P < 0.001 \)) concentrations, compared with control values (Fig. 10A). In contrast, AG1024 had no effect on the growth of the
Figure 6 (A) Initial inhibition of TAM-R cell invasion by gefitinib is circumvented in the drug-resistant breast cancer cell line. TAM-R cells were grown in the absence and presence of 1 μM gefitinib in routine medium and TAM/TKI-R cells were grown in their routine medium. After 4 days, the cells were trypsinised and re-seeded onto Matrigel-coated porous membrane filters and cultured for 72 h in the identical conditions prior to re-seeding. After fixing and staining with DAPI, numbers of migrated cells were assessed by fluorescent microscopy. The graph illustrates the number of invaded cells per membrane and represents the means ± S.D. of three experiments. (B) The IGF-1R inhibitor AG1024 reduces the invasive capacity of TAM/TKI-R cells. The TAM-R (solid bars) and TAM/TKI-R cells (spotted bars) were grown in routine culture medium for 4 days, subsequently re-seeded onto Matrigel in medium containing 0–10 μM AG1024 and cultured and assessed as detailed in (A). Results are expressed as mean numbers ± S.D. of invaded cells of triplicate membranes from one experiment and represent three individual experiments. *P ≤ 0.05.
parental DU145 cell line up to 10 μM concentration (P = 0.895) (Fig. 10A).

**Invasive capacity**
The migratory potential of the DU145 and DU145/TKI-R cell lines were investigated using an *in vitro* invasion assay. Considerable and equivalent numbers of cells from both cell cultures invaded through the Matrigel and adhered to the underside of the porous membrane after 72 h of culture (Fig. 10B). It was seen that, compared with control values, AG1024 had no effect on the migration of DU145 cells (P = 0.864; Fig. 10B). In contrast, it was observed that at a concentration of 10 μM the IGF-1R inhibitor AG1024 significantly reduced (45%, P ≤ 0.05) the migratory ability of the DU145/TKI-R cells, compared with control values (Fig. 10B).

**Discussion**
Clinical data have already demonstrated that the prevention of EGFR-mediated signal transduction by the small molecule inhibitor gefitinib provides a promising new treatment option for a variety of cancer types (Barton *et al.* 2001, Cohen *et al.* 2002, Fukuoka *et al.* 2002, Goss *et al.* 2002, Herbst & Kies 2002, Ranson 2002). Such studies have also indicated, however, that responses to gefitinib were heterogeneous with not all patients responding to treatment and, even in responders, disease relapse was inevitable (Barton *et al.* 2001, Cohen *et al.* 2002, Fukuoka *et al.* 2002, Goss *et al.* 2002, Herbst & Kies 2002, Ranson 2002). The data thus documented the existence of *de novo* resistance and the acquisition of resistance to the drug. In our study, we have generated models for acquired resistance to gefitinib using an EGFR-positive, tamoxifen-resistant, MCF-7-derived breast cancer cell line (Knowlden *et al.* 2003) and the androgen-independent, EGFR-positive prostate cancer cell line DU145. Both these cell cultures showed substantial growth inhibition when initially challenged with 1 μM gefitinib. The surviving population, however, eventually resumed proliferation even though successful blockade of EGFR signal transduction was demonstrated in these cells. It is an interesting observation that resistance to gefitinib developed slowly in the breast cancer cells compared with the more rapid generation of tamoxifen resistance, i.e. 6 months and 2–3 months respectively, implying that other growth-regulatory pathways are not readily available to these cells in the absence of EGFR signalling.
Figure 8 DU145/TKI-R cells show elevated levels of components of the IGF-1R signalling pathway compared with their WT parents. (A) RT-PCR analysis of IGF-II mRNA production in the DU145 and DU145/TKI-R cells assessed after 7 days growth in routine culture medium. In addition, levels of bFGF mRNA were also determined. Data represent three analyses. (B) DU145 and DU145/TKI-R cells were grown in routine culture medium for 7 days. Cell lysate was electrophoresed, immunblotted for total IGF-1R and phosphorylated (phospho-) IR/IGF-1R<sup>Y1159/pY1131</sup>, PKCδ and ERK1/2 and results were normalised to β-actin levels as detailed in Fig. 1B. The data illustrated are representative of three separate experiments.
Our data suggest, however, that IGF-1R signalling may be one of the mechanisms that can compensate for the long-term suppression of EGFR function in both breast and prostate cancer cells.

The TAM/TKI-R breast cancer cells showed substantial growth promotion following challenge with IGF-I and IGF-II and were clearly more responsive to these ligands than the parental TAM-R cells. Additionally, the TAM/TKI-R cells were also stimulated by bFGF and heregulin-β. Of these mitogenic growth factors, however, only IGF-II mRNA was detected in the TAM/TKI-R cells and in amounts similar to that seen in the parental line. In addition, a significant increase in basal IGF-1R phosphorylation was demonstrated in the gefitinib-resistant population compared with parental cells. Furthermore, the enhanced role postulated for the IGF-1R in

![Image of immunocytochemical localisation of total IGF-1R](A) DU145 and (B) DU145/TKI-R cells and phosphorylated IGF-1R in (C) DU145 and (D) DU145/TKI-R cell lines after 7 days growth in routine culture medium.

<table>
<thead>
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<th>Mean basal H-score</th>
<th>DU145</th>
<th>DU145/TKI-R</th>
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<tr>
<td>Total IGF1-R</td>
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<tr>
<td>Cytoplasmic</td>
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<td>Membrane</td>
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<td>Total</td>
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<td>Phospho-IGF1-R</td>
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<td>Cytoplasmic</td>
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<tr>
<td>Total</td>
<td>119±9</td>
<td>206±6</td>
</tr>
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</table>

Table 2 H-score analysis of immunocytochemistry performed on day 7 for total and phosphorylated (phospho-) IGF-1R in DU145 and DU145/TKI-R cells. Data represent the means ± S.E.M. of the assessment of a minimum of two fields/cover-slip (with three cover-slips/treatment).
Figure 10  (A) DU145/TKI-R cells show increased sensitivity to treatment with the IGF-1R inhibitor AG1024 compared with their parental cells. Growth analysis of DU145 (solid bars) and DU145/TKI-R (spotted bars) cells grown in routine culture medium in the absence and presence of AG1024 (1–10 μM). Results are expressed as % of control values and show the means ± s.d. of triplicate wells. The data are representative of three individual experiments. *P ≤ 0.05. (B) DU145/TKI-R cell migration is reduced by AG1024 treatment. For invasion studies, WT DU145 and DU145/TKI-R cells were grown in the absence and presence of AG1024 (1–10 μM) in routine medium for 4 days. The cells were trypsinised and re-seeded onto Matrigel-coated porous membrane filters and cultured for 72 h in identical conditions prior to re-seeding. After fixing and staining with DAPI, numbers of migrated DU145 (solid bars) and DU145/TKI-R cells (spotted bars) were assessed by fluorescent microscopy. Results are expressed as mean numbers ± s.d. of invaded cells of triplicate membranes from one experiment and represent three individual experiments. *P ≤ 0.05.
mediating the growth of TKI-R breast cancer was reinforced by dose-response studies, in which significant differences in sensitivity to the IGF-1R TKI AG1024 were shown in the TK1-R cell line compared with the parental line. The role of IGF-1R in mediating cell proliferation in acquired resistance to gefitinib was investigated further in the DU145/TKI-R prostate cell line. Compared with the parental WT cells, considerably higher basal levels of IGF-II mRNA were demonstrated in the DU145/TKI-R cells, together with significant increases in total and membrane-associated phosphorylated IGF-1R, suggesting activation by classical autocrine/paracrine signalling in this phenotype. Furthermore, the gefitinib-resistant prostate cells were markedly sensitive to the IGF-1R inhibitor AG1024, with no effect on parental DU145 cell growth being observed up to 10 μM concentration. Exogenous IGF-I and IGF-II failed to induce either proliferation or additional ligand-induced activation of the IGF-1R over basal levels in the WT DU145 and, paradoxically, DU145/TKI-R cells. This may be interpreted as first, in the WT cells IGF-1R signalling is not a key growth pathway and secondly, given the high basal growth rate and elevated levels of phosphorylated IGF-1R demonstrated by the DU145/TKI-R cells, further increases in growth or receptor activation respectively would be difficult to promote. The importance of IGF-1R signalling in the DU145/TKI-R cells is, however, indicated by the sensitivity of the DU145/TKI-R cells to inhibition of the IGF-1R.

Clearly, the prostate and breast gefitinib-resistant phenotypes have compensated for EGFR blockade via IGF-1R signalling; however, the models differ considerably in their dependence and cross-talk between the EGFR and IGF-1R pathways. The DU145/TKI-R prostate cells increased IGF-1R and IGF-II and sensitivity to IGF-1R inhibition, whereas the parental DU145 cells were unaffected by the IGF-1R inhibitor. Furthermore, although the EGFR inhibitor reduced initial WT DU145 cell growth by 60%, these cells did not show any growth inhibition by the IGF-1R inhibitor. On the acquisition of resistance to the EGFR inhibitor, however, they acquired approximately 50% growth inhibition in response to inhibition of the IGF-1R. This implies that there is no cross-talk between these pathways in the prostate cells and merely a switch in pathways with the intervention described. Other workers have shown, however, that short-term response to IGF in DU145 cells depends upon an intact EGF/IGF-α autocrine loop as assessed by the blocking effect of a specific EGFR antibody (Connolly & Rose 1994) and therefore one would logically predict that long-term suppression of EGFR signalling would diminish the importance of IGF-1R-mediated events which is clearly not the case in our study. It is possible that the interplay between the two receptors may alter with the long-term EGFR inhibition and that, in the DU145 cells, EGFR and IGF-1R cross-talk has become uncoupled with chronic acquired gefitinib resistance. Such data support the importance of the utilisation of chronic exposure models in order to delineate mechanisms of acquired drug resistance as they may reflect the clinical scenario more closely than acute drug challenge.

In contrast to the prostate model, the TAM/TKI-R breast cancer cells had only a modest increase in both IGF-1R phosphorylation and sensitivity to the IGF-1R inhibitor compared with the parental TAM-R cells. This indicated that the TAM-R cells were also dependent upon IGF-1R signalling which was revealed when the EGFR pathway was blocked and therefore reflected the existence of potential cross-talk between the two pathways. Our in vitro studies have shown that, in the TAM-R cells, levels of IGF-1R expression/activation recovered substantially following the considerable 80% reduction in receptor levels seen after the initial treatment of the TAM-R parental MCF-7 cells with tamoxifen (Nicholson et al. 2004). Thus, although EGFR-mediated growth is critical in these cells, they also appear to require activation of the IGF-1R. Furthermore, in the TAM-R cells, it appears that the IGF-1R is permissive for EGFR signalling as increases in the phosphorylation of the IGF-1R and subsequently the EGFR were seen following stimulation with IGF-II, events which could be blocked with AG1024 (Nicholson et al. 2004). Indeed, IGF-1R/EGFR cross-talk has been reported in several tumour cell types (Roudabush et al. 2000, Gilmore et al. 2002, Wang et al. 2002). It is conceivable that in this TKI-R breast cancer phenotype, increased receptor activation may be achieved via transphosphorylation of the IGF-1R in cells overexpressing the receptor (Yoshinouchi et al. 1993). No bFGF, however, was detected in TAM/TKI-R breast cells. Nevertheless, it is possible that the increased production of other (unknown) growth factors may have accounted for transphosphorylation of the IGF-1R. Alternatively, it has been shown that a reduction in the activity of SHP-2, a cytoplasmic tyrosine phosphatase with two Src-homology 2 (SH-2) domains, can lead to more stable phosphorylation of IGF-1R (Maile & Clemmons 2002).

Our studies have indicated that IGF-1R-mediated growth/signalling plays an important role in the maintenance of cell proliferation in the gefitinib-resistant cells. Indeed, recent evidence has also shown that an association exists between up-regulated IGF-1R signalling and resistance to drugs which inhibit erbB family signal...
transduction. For example, IGF-1R signalling has been shown to be central in modulating the responses to trastuzumab, a humanised antibody directed against the TK receptor HER-2 (erbB-2, neu). It was seen that trastuzumab inhibited the growth of MCF-7/HER-18 cells, which over-express HER-2 receptors and express IGF-1R, only when IGF-1R signalling was minimised (Lu et al. 2001). IGF-1R has also been shown to mediate resistance to anti-EGFR therapies in glioblastoma cells (Chakravati et al. 2002) and in the DiFi human colorectal cancer cell line (Liu et al. 2001).

It is established that the consequences of IGF-1R activation by its ligands result in the recruitment of major adapter signalling proteins such as src/collagen homology proteins (SHC) which lead to interaction with Grb/SOS and ultimately RAS/MAPK (ERK) signalling cascades and insulin receptor substrate-1 leading to PI3-kinase/AKT activation (Sachdev & Yee 2001). Interestingly, it was observed that ERK1/2 activity appeared to be greatly diminished with chronic exposure to the EGFR inhibitor despite increased activation of the IGF-1R. Together with the findings which showed that the inhibition of IGF-1R in the TAM-R cells resulted in the decreased activation of ERK1/2, whereas ERK1/2 activity was unaffected by the IGF-1R inhibitor in the TAM/TKI-R cells, suggested that ERK1/2 had become uncoupled from the IGF-1R during the manipulation of these cells. Other studies, however, have demonstrated that PKCδ, a member of the PKC isoform family, known to be involved in the regulation of various cellular functions such as proliferation, differentiation and cell survival (Dekker & Parker 1994), can be a direct tyrosine substrate for IGF-1R (Li et al. 1998). Furthermore, both protein and mRNA levels of this kinase have been shown to be up-regulated upon long-term IGF-1R activation (Li et al. 1998). Our current study has demonstrated that both the breast and prostate gefitinib-resistant phenotypes possess elevated levels of phosphorylated PKCδ and, additionally, the breast cancer gefitinib-resistant cell line also contained elevated basal levels of phosphorylated AKT. Furthermore, in the TAM/TKI-R cells, stimulation of the IGF-1R by IGF-I and IGF-II led to a considerable increase in PKCδ and AKT activity, whereas in the TAM-R cells the only notable change was a moderate increase in phosphorylated AKT after exposure to IGF-I. The results suggest that PKCδ and AKT are downstream targets for the IGF-1R in the TAM/TKI-R cells and, furthermore, imply a difference in the usage of IGF-II between these cell lines. Moreover, the activity of both PKCδ and AKT was modulated by exposure to AG1024, further supporting the indication that they are potential downstream targets for IGF-1R signalling.

It is also known, however, that PKCδ can be phosphorylated by the 3-phosphoinositide-dependent protein kinase-1 which is downstream of PI3 kinase (Le Goode et al. 1998, Alessi 2001). The importance of and the underlying mechanisms involving AKT and PKCδ in mediating proliferation under the influence of IGF-1R is currently under investigation. It is noteworthy that phosphorylated AKT could not be detected in the prostate gefitinib-resistant cells, although this is not surprising as the cell line was maintained in serum-free medium and it has been reported previously that under these conditions DU145 cells contain negligible amounts of phosphorylated AKT (Klein & Fischer 2002).

An interesting observation in the current study was the non-EGFR-mediated invasive capacity of the gefitinib-resistant breast cancer cells. Initially, the invasiveness of the EGFR-positive TAM-R cells was considerably reduced by treatment with the inhibitor. Indeed, EGFR over-expression/activation is known to promote cancer cell metastasis and studies have indicated that EGFR-mediated invasion can be inhibited by targeting this receptor (Xie et al. 1995, Genersch et al. 1998, Shao et al. 1998, Harper et al. 2002). In the current study, however, the TAM/TKI-R cells were able to override the initial inhibitory effects of gefitinib on invasion and went on to develop an invasive capacity far exceeding that of their TAM-R parent. Similarly, we have demonstrated previously that DU145 cell invasion can be diminished by EGFR inhibition (Harper et al. 2002) and, as seen in the TAM/TKI-R cells, the DU145/TKI-R cell line also showed the ability to bypass the initial inhibition of invasion by gefitinib and regenerate substantial migratory capabilities. The IGF-1R has been linked with the modulation of the metastatic potential of tumour cells (Yu & Rohan 2000, Brodt et al. 2001) and it was observed that treatment with the IGF-1R inhibitor AG1024 reduced cell migration in the gefitinib-resistant breast and prostate cancer cell lines but not their parent cells, indicating that IGF-1R expression/signalling played a role in the migratory ability of these variant cell lines.

In summary, gefitinib has effective anti-tumour activity in a range of EGFR-positive cancer types but resistance to treatment has also been demonstrated. Elucidation of the cellular mechanisms that result in resistance to the inhibitor is essential with regard to the development of drug therapies that target these signalling molecules. Our study has demonstrated an important role for IGF-1R in the mediation of cell growth and invasion in the acquired gefitinib-resistant phenotype. Thus, therapeutic strategies that target the IGF-1R may increase the efficacy and duration of response to gefitinib.
Acknowledgements

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