Molecular aspects of gefitinib antiproliferative and pro-apoptotic effects in PTEN-positive and PTEN-negative prostate cancer cell lines

C Festuccia¹, P Muzi¹, D Millimaggi¹, L Biordi¹, G L Gravina², S Specia¹, A Angelucci², V Dolo¹, C Vicentini² and M Bologna¹,³

Departments of ¹Experimental Medicine, ²Surgery and ³Basic and Applied Biology, University of L'Aquila, Via Vetoio, Coppito-2, 67100 L'Aquila, Italy

(Requests for offprints should be addressed to C Festuccia; Email: festucci@univaq.it)

Abstract

To date, no effective therapeutic treatment allows abrogation of the progression of prostate cancer (PCa) to more invasive forms. One of the major targets for the therapy in PCa can be epidermal growth factor receptor (EGFR), which signals via the phosphoinositide 3'-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways, among others. Despite multiple reports of overexpression in PCa, the reliance on activated EGFR and its downstream signalling to the PI3K and/or MAPK/extracellular signal-regulated kinase (ERK) pathways has not been fully elucidated. We reported that the EGFR-selective tyrosine kinase inhibitor gefitinib (ZD1839; Iressa) is able to induce growth inhibition, G1 arrest and apoptosis in PCa cells and that its effectiveness is associated primarily with phosphatase and tensin homologue deleted from chromosome 10 (PTEN) expression (and thus Akt activity). In fact PTEN-negative PCa cells are slowly sensitive to gefitinib treatment, because this molecule is unable to downregulate PI3K/Akt activity. PI3K inhibition, by LY294002 or after PTEN transfection, restores EGFR-stimulated Akt signalling and sensitizes the cells to pro-apoptotic action of gefitinib. The MAPK pathway seems to be involved primarily on cell-growth modulation because dual blockade of EGFR and ERK1/2 phosphorylation potentiates growth inhibition (both not cell apoptosis) in PTEN-positive PCa cells and reduced EGF-mediated growth in PTEN-negative cells. Thus the effectiveness of gefitinib requires growth factor receptor-stimulated PI3K/Akt and MAPK signalling to be intact and functional. The loss of the PTEN activity leads to uncoupling of this signalling pathway, determining a partial gefitinib resistance. Moreover, gefitinib sensitivity may be maintained in these cells through its inhibitory potential in MAPK/ERK pathway activity, modulating proliferative EGFR-triggered events. Therefore, our data suggest that the inhibition of EGFR signalling can result in a significant growth reduction and increased apoptosis in EGFR-overexpressing PCa cells with different modalities, which are regulated by PTEN status, and this may have relevance in the clinical setting of PCa.

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Introduction

Prostate cancer (PCa) is presently the second leading cause of cancer-related death and the most commonly diagnosed non-skin cancer in men. To date, no effective therapeutic treatment allows the abrogation of its progression to more invasive disease forms. In fact, while PCa is frequently curable in its early stage, approximately one-third of PCa patients present with advanced disease that requires development of novel therapeutic approaches for its control and to improve patient survival. The primary focus of prostate research programs is to understand the molecular mechanism(s) that govern the progression of PCa from a state of androgen sensitivity to hormone independence with the hope of developing novel
therapeutic strategies to prevent or delay the progression of PCa to androgen independence, and with the aim of designing targeted therapies.

A central regulator of human PCa progression seems to be the phosphatase and tensin homologue deleted from chromosome 10 (PTEN) tumour-suppressor gene, which maps to 10q23 (reviewed by Osaki et al. 2004). PTEN gene is inactivated in ~50% of prostate tumours and is correlated with advanced disease and a poor prognosis (Dreher et al. 2004, Pfeil et al. 2004) and Akt activation is probably important for the progression of PCa to an androgen-independent state. As PTEN encodes a lipid phosphatase that is a negative regulator of the phosphoinositide 3'kinase (PI3K)/Akt pathway, loss of function for PTEN results in inappropriate activation of Akt and continuous signalling through the PI3K pathway as a result of PTEN deficiency confers protection of PCa cells from cell death and resistance to chemotherapy (reviewed in Wang et al. 2004). However the key role of the PI3K/Akt in promoting cell survival has been well characterized (Brader & Eccles 2004, Stahl et al. 2004). In addition, PTEN loss-of-function mutations result in homzygous embryonic lethality, whereas heterozygotes have dysplasia and/or carcinoma of many tissues, including prostate (Kim et al. 2002, Abate-Shen et al. 2003, Mazzucchelli et al. 2004). Nkx3.1+/−; Pten+/− mice develop several key features of the advanced stages of PCa, including invasiveness, the potential for androgen independence and metastases, and a critical dependence on aging for disease progression. In addition, high levels of activated mitogen-activated protein kinase (MAPK) were found in recurrent tumour samples after hormonal therapy (Gioeli et al. 1999).

Two epidermal growth factor (EGF) receptor (EGFR) family members, EGFR (Erb-B1) and Her2 (Erb-B2), are frequently overexpressed in PCa and this is associated with a more aggressive clinical outcome (Di Loreto et al. 2002). In addition, EGFR/Her2 and its ligands, EGF and transforming growth factor α (TGFα), play a critical role during tumourigenesis of the prostate gland (CuliC et al. 1996, Itoh et al. 1998) and EGFR signalling has been linked to the progression of androgen-responsive PCa to androgen-independent/hormone refractory tumours (Djakiew 2000, El Sheikh et al. 2004, Gravina et al. 2004, Festuccia et al. 2005). 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 EGF and TGFα (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, Festuccia et al. 2005). The EGFR or Erb-B1 is a proto-oncogene, which encodes a 170 kDa protein that consists of an extracellular ligand-binding domain, a transmembrane domain, an intracellular tyrosine kinase domain and a C-terminal regulatory domain containing sites of autophosphorylation. Upon binding of its ligands, EGFR will undergo homo- and/or heterodimerization with other members of this receptor family including Erb-B2, Erb-B3 and Erb-B4, resulting in addition of phosphate moieties to specific tyrosines, which can serve as docking sites for downstream effectors. This triggers a cascade of biochemical and physiological responses that constitute the mitogenic signal transduction of the cells. Several kinase cascades have been implicated in signal transduction through the Erb-B receptors including the Ras/Raf and PI3K/Akt pathways.

Given the frequency at which abnormalities in EGFR signalling are present in malignant PCa and the demonstrated roles of these changes in the transformation and production of phenotypes associated with increased malignancy of PCAs, this receptor is an attractive target for therapeutic manipulation. Gefitinib (Iressa) is an oral anilinoquinazolone compound that inhibits the tyrosine kinase activity of the EGFR (Ciardiello et al. 2000, Arteaga & Johnson 2001), resulting in the inhibition of the activation of downstream signalling molecules.

Gefitinib presents much therapeutic promise for the treatment of numerous human cancer types; however, the clinical data demonstrate that not all patients respond to the inhibitor, indicating the existence of 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). PTEN absence has been associated with gefitinib resistance in several cancers (She et al. 2003) and basal activation of extracellular signal-regulated kinase (ERK) 1/2 seems to prevent the effectiveness of EGFR inhibitors in breast cancer cells (Ono et al. 2004).

Gefitinib inhibits the growth of cell lines that express high levels of both EGFR and Her2 (Vicentini et al. 2003), induces complete regression of well-established xenografts (Ciardiello et al. 2000, Sirotnak et al. 2002, Asakuma et al. 2004) and has recently entered clinical trials in cancer patients (Lorusso 2003, Natale 2004, Wolf et al. 2004). Intrinsic and acquired resistance to chemotherapy is a major obstacle to successful cancer treatment. Understanding the mechanisms by which
tumours become resistant to a particular agent is key to identifying new drugs or combination regimens. 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 PCa xenografts (Geller et al. 2002). Development of resistance has also been described with other signal transduction inhibitors such as trastuzumab (Herceptin). Resistance to this human antibody directed against the extracellular domain of Erb-B2, used in the treatment of Erb-B2-positive metastatic breast cancer, has been reported to occur within 12 months (Baselga 2001, Slamon et al. 2001). It is possible that resistance to gefitinib may be acquired by the ability of the tumour cells to utilize alternative growth factor pathways in the presence of the drug. EGFR-overexpressing MDA-MB468 breast cancer cells lacking PTEN function are resistant to gefitinib (She et al. 2003), and this drug is unable to downregulate Akt activity in these cells while pharmacologic downregulation of constitutive PI3K/Akt pathway signalling using the PI3K inhibitor LY294002 restores EGFR-stimulated Akt signalling and sensitizes cells to gefitinib. These results suggested that sensitivity to gefitinib requires intact growth factor receptor-stimulated Akt signalling activity.

Our study determined whether there is a correlation between the effectiveness of gefitinib and EGFR/Her2 status, and PI3K or MAPK activity in PCa cells, and how this affects the response to an inhibitor of the receptor. We reported that PTEN-negative PCa cells are only partially resistant to gefitinib treatment because this molecule is unable to downregulate PI3K/Akt activity in these cells. Comparing PTEN-negative with PTEN-positive cells we observed that exposure to gefitinib induced differential patterns of cell-cycle arrest and apoptosis features as well as distinct patterns of effects on Akt, ERK1/2, c-Jun N-terminal kinase (JNK) 1/2 and p38 MAPK phosphorylation.

Materials and methods
Reagents
All the materials for tissue culture were purchased from Hyclone (Cramlington, NE, USA). Plasticware was obtained from Nunc (Roskilde, Denmark). EGF was purchased from Immuno Tools GmbH (Friesoythe, Germany). Gefitinib was obtained from AstraZeneca. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) unless otherwise indicated. PTEN antibody has been purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies against phosphorylated forms of EGFR, HER2 and ERK1/2 were obtained from Biosource International (Camarillo, CA, USA). Akt/protein kinase B (PKB) kinase activity was performed using a non-radioactive assay kit (StressXpress AKT/PKB Elisa kit) which was purchased from Stressgene Bioreagents (Victoria, BC, Canada) on cell lysates after partial purification on a MonoQ exchange column (2 mg protein in 1 ml column) with 10 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 2 mM sodium orthovanadate and 2 mM dithiothreitol and eluted using 12 ml linear NaCl gradient (0–0.8 M NaCl) with a flow rate of 0.5 ml/min. We collected fractions between 0.25 and 0.5 ml for analysis.

Cell lines
We used three androgen-sensitive PCa cell lines (ND1, LnCaP and CWR22R-2152 xenograft-derived) and four androgen-insensitive PCa cell lines (PC3, DU145, ALVA31 and TSU-Pr1). CWR22R-2152 cell cultures were obtained in our laboratory from a xenografted CWR22 relapsed tumour (Tepper et al. 2002) kindly provided by Dr T Pretlow (Case Western Reserve University, Cleveland, OH, USA). LnCaP, PC3 and DU145 cell lines were originally obtained from ATCC (Rockville, MD, USA). ND1 cells (Narayan & Dahiy 1992) were kindly provided by Dr R Dahiy (University of California, San Francisco, CA, USA). TSU-Pr1 cells (Izumi et al. 1987) were kindly provided by Dr K Takeda (Science University of Tokyo, Tokyo, Japan) and ALVA31 cells (Loop et al. 1993) were kindly provided by B A Roos (Univerity of Miami, Coral Gables, FL, USA). PTEN-transfected PC3 cells (Zhao et al. 2004) were kindly provided by Dr D LeRoith (National Institutes of Health, Bethesda, MD, USA).

Reverse transcriptase (RT)-PCR for PTEN expression
Total RNA was extracted from PCa cells lines with Trizol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer’s instructions. An RT-PCR was carried out on DNase Amp Grade (Gibco-BRL)-treated RNA using Murine Leukaemia Virus (MuLV) RT (50 U) in 100 mM Tris/HCl, pH 8.3,
500 mM KCl, 5 mM MgCl₂, 0.5 μM each dNTP, 1 U RNasin and 500 pmol random examer primers. cDNA (2 μl) were amplified in a 50 μl reaction volume containing 0.5 μM sense and antisense primers, 2.5 U Taq DNA polymerase (Applied Biosystems, Milan, Italy), 200 μM each dNTP and 1.5 mM MgCl₂. A co-amplification of GAPDH was performed. Primer sequences used for PCR were: PTEN 3rd exon, forward, 5'-ATATTCTCTGAAAGCTCTGG-3’, and reverse, 5’-TTAATCGGTATGGAATCAA-3’; GAPDH, forward, 5’-CACCATGGAGAAAGGCCGGGGG-3’, and reverse, 5’-GACGGACACATTGGGGGATGGTAG-3’. After amplification, 20 μl of the PCR reaction mixture was analysed by 1.2% agarose gel electrophoresis and stained with ethidium bromide.

**Growth assays**

Cells were seeded at a density of 2 × 10⁴ cells per dish on 50 mm petri dishes. Cells were left to attach and grow in 5% fetal calf serum/Dulbecco’s modified Eagle’s medium (DMEM) for 24 h. After this time, cells were maintained in culture medium containing androgens or subjected to androgen depletion. All other cells were treated with either 50 ng/ml EGF or different doses of gefitinib and/or inhibitors of single molecules involved in EGFR-mediated signalling (LY294002, a PI3K inhibitor; PD98059 and U0126, MAPK/ERK kinase (MEK) inhibitors; SB203580, a p38 MAPK inhibitor; and SP600125, a JNK inhibitor) at the recommended inhibition doses. Growth assays were performed in triplicate. To evaluate the effective counts were performed for each dish. All experiments were conducted in triplicate. To evaluate the effective cell proliferation we measured the uptake of [³H]thymidine. Briefly, PCa cells ((1–2) × 10⁴ cells/well) were grown overnight in 24-well plates and exposed to either gefitinib (0.1–10 μM) or DMEM (control). After treatment, cells were pulsed with [³H]thymidine (1 μCi/well) for 4–6 h, fixed with 5% trichloroacetic acid and solubilized in 0.5 M NaOH before scintillation counting. Experiments were performed in triplicate.

**Assessment of thymidine incorporation in gefitinib-treated PCa cells**

Cell-proliferation studies were performed by measuring the uptake of [³H]thymidine. Briefly, PCa cells ((1–2) × 10⁴ cells/well) were grown overnight in 24-well plates and exposed to either gefitinib (0.1–10 μM) or DMEM (control). After treatment, cells were pulsed with [³H]thymidine (1 μCi/well) for 4–6 h, fixed with 5% trichloroacetic acid and solubilized in 0.5 M NaOH before scintillation counting. Experiments were performed in triplicate.

**Preparation of cell lysates and western blot analysis**

Following treatment, cells were washed with cold PBS and immediately lysed with 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM p-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 μg/ml aprotonin and 10 μg/ml leupeptin). Lysates were electrophoresed by 7% SDS/PAGE, and separated proteins transferred to nitrocellulose and probed with the appropriate antibodies using the conditions recommended by the suppliers. EGFR and ERK activation status was analysed using phosphospecific antibodies, and blots were stripped and reprobed with the appropriate antibody for assessment of total ERK or EGFR expression.

**Immunofluorescence analysis**

Quantification of EGFR/HER2-positive cells was performed by flow-cytometric analysis (FACScan; Becton Dickinson, Mountain View, CA, USA). Cells were trypsinized, centrifuged and left at 37°C for 1 h in DMEM/10% fetal calf serum in polypropylene tubes to reconstitute cellular external membrane. Cells were washed in saline buffer and 1 × 10⁶ cells were fixed in a 3.7% paraformaldehyde-buffered solution, washed twice and treated with about 10 μg/ml primary antibodies. After 1 h at 4°C cells were washed twice in PBS and FITC-conjugated anti-rabbit and anti-mouse secondary antibodies (about 1 μg/ml) were added to the fixed cells. After 30 min of incubation at 4°C, cells were washed twice and resuspended in PBS at 1 × 10⁶ cells/ml before analysis using Cell Quest software (Becton Dickinson).
Cell-cycle and apoptosis analysis

The adherent cells were trypsinized, pooled with the culture supernatant containing the apoptotic cells already detached from the dish and centrifuged. Cells \((1 \times 10^6)\) were washed in PBS and fixed for 30 min by the addition of 1 ml 70% ethanol. After 30 min, the cells were pelleted by centrifugation \((720 \times g; 5 \text{ min})\), and resuspended in 1 ml of DNA staining solution \((\text{PBS containing } 200 \text{ mg/ml RNase A, } 20 \text{ mg/ml propidium iodide and } 0.1\% \text{ Triton X-100})\) and stained by incubation at room temperature for 60 min. All cells were then measured on a FACScan flow cytometer with an argon laser at 488 nm for excitation and analysed using Cell Quest software. All flow-cytometric measurements were made using the same instrument settings, and at least 10,000 cells were measured in each sample. Apoptotic cells were detected by a quantifiable peak in sub-G\(_1\) phase corresponding to the red fluorescent light emitted by sub-diploid nuclei of cells, and the results were expressed as the percentage of death by apoptosis induced by a particular treatment.

Statistics

Statistical analysis was performed using SPSS 11.0 software \((\text{SPSS, Chicago, IL, USA})\). All \(P\) values less than 0.05 were considered to indicate significance. All statistical tests were two-tailed. Differences in ordinary variables were compared with \(\chi^2\) test for \(2 \times 2\) tables or Fisher’s exact test when the tables were too sparse. Continuous variables were analysed using the Wilcoxon rank sum test. Whenever we correlated a continuous variable with a dichotomy or an ordinal variable, as a measure of the strength of a relationship, we used the Point-biserial correlation.

Results

Antiproliferative effects of gefitinib

We analysed in PCa cells the expression of (1) PTEN, by western blotting and RT-PCR, (2) phospho- (p-) Akt and Akt activity, by western blotting and Akt/PKB kinase activity assay kit, (3) p-ERK1/2 by western blotting, and (4) EGFR and Her2, by FACS analysis. In addition we compared the levels of the above-mentioned parameters with gefitinib effectiveness as shown in Fig. 1 and Table 1.

RT-PCR analysis revealed that PC3, ALVA31 and TSU-Pr1 cells do not express PTEN mRNA whereas ND1, LnCaP, CWR22R-2152 and DU145 cells were PTEN-positive (Fig. 1A). Western blot analysis revealed that ND1, DU145 and CWR22R-2152 cells express PTEN protein whereas LnCaP cells, in agreement with some reports, do not express this protein (Fig. 1B). PTEN protein-negative cells possess higher levels of activated (phosphorylated) Akt (p-Akt) compared with PTEN protein-positive cells (Fig. 1B and C).
PTEN expression was evaluated by both RT-PCR and western blotting analysis, thus — represents the absence and + the presence of PTEN. LnCaP cells contain detectable levels of mRNA for PTEN but not of PTEN protein (therefore we used the ± symbol). Akt/PKB activity was evaluated by ELISA test and the values represent the ng of active protein present in the test.

Next we verified whether gefitinib effectiveness was associated with p-ERK1/2 basal levels. To compare the levels of basal ERK1/2 activation in each tested cell line, we quantified the activation of ERK1/2 (p-ERK) densitometrically. Densitometry of immunoreactive bands showed the phosphorylation of ERK1/2 with respect to total ERK. Immunoblotting revealed differences in the basal levels of ERK1/2 phosphorylation in different PCA cell lines, whereas the expression of ERK1/2 protein, normalized to actin expression, was relatively consistent, as shown in Fig. 1D. The IC₅₀ values for gefitinib treatment were statistically different (0.74 ± 0.28 versus 0.55 ± 0.35 μM, respectively) and PTEN-transfected PC3 cells (Zhao et al. 2004) were more sensitive than wild-type PC3 cells (0.25 versus 0.70 μM). These results indicate that the drug’s antiproliferative effectiveness, evaluated by IC₅₀ value, could be not completely dependent on PTEN function. In contrast, the correlation between the effectiveness of gefitinib and basal Akt activity revealed r = 0.709 (P = 0.049; Fig. 1C and Table 1), suggesting that the elevated basal Akt activity was a negative parameter for gefitinib efficacy.

Next we compared gefitinib effectiveness with Her2 levels (Table 1). The analysis of the correlation revealed that gefitinib effectiveness was independent for Her2 levels (r = 0.594; P = 0.12). However, the effects of gefitinib were most evident in cells with high levels of EGFR and high basal levels of phosphorylated ERK1/2 (DU145 cells).

As previously observed (Vicentini et al. 2003) gefitinib induced a dose-dependent G₀/G₁ proliferative arrest with apoptosis induction in PCA cells. We observed that the percentage of apoptotic cells, measured at 48 h in the presence of 0.5 μM gefitinib, is considerably lower in PTEN-negative than PTEN-positive cells (11.0 ± 6.0 versus 33.0 ± 6.0%, respectively; P < 0.05), whereas no statistical significance was demonstrated when we compared apoptosis with ERK activation. A negative correlation was observed comparing Akt activity and gefitinib-induced apoptosis (r = −0.828; P < 0.001), suggesting that the elevated Akt activity is protective for gefitinib-induced cell apoptosis.

Taken together, the data illustrated in this section indicate that MAPK activity modulates primarily the antiproliferative effects of gefitinib whereas PTEN modulates the pro-apoptotic activities of this drug.

C Festuccia et al.: Molecular mechanisms in Gefitinib antiproliferative action

Table 1 Characterization of the eight tested PCA cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PTEN</th>
<th>Akt/PKB</th>
<th>p-Erk</th>
<th>EGFR</th>
<th>Her2</th>
<th>IC₅₀</th>
<th>Gefitinib</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>−</td>
<td>5.5</td>
<td>1.5</td>
<td>4.8</td>
<td>3.0</td>
<td>0.70</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>PC3-PTEN</td>
<td>+</td>
<td>0.1</td>
<td>0.6</td>
<td>4.0</td>
<td>3.0</td>
<td>0.25</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>ALVA31</td>
<td>−</td>
<td>7.8</td>
<td>4.0</td>
<td>3.0</td>
<td>5.7</td>
<td>0.89</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>TSU-Pr1</td>
<td>−</td>
<td>10.4</td>
<td>5.2</td>
<td>1.5</td>
<td>7.0</td>
<td>1.0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>DU145</td>
<td>+</td>
<td>0.3</td>
<td>2.0</td>
<td>9.0</td>
<td>4.0</td>
<td>0.55</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>ND1</td>
<td>+</td>
<td>0.1</td>
<td>3.0</td>
<td>2.5</td>
<td>1.5</td>
<td>0.90</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>CWR22R-2152</td>
<td>+</td>
<td>0.1</td>
<td>1.7</td>
<td>5.9</td>
<td>2.0</td>
<td>0.20</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>LnCaP</td>
<td>±</td>
<td>1.5</td>
<td>0.8</td>
<td>2.0</td>
<td>1.5</td>
<td>0.37</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>
PC3 and DU-145 cells have distinct PTEN expression and Akt activation

In Fig. 2 we show that PTEN-positive (DU145 cells; Fig. 2A) and PTEN-negative (PC3 cells; Fig. 2B) cells have basally different levels of the activated form of Akt (p-Akt). EGF and serum increased Akt activation only in DU145 cells. In serum-free conditions PC3 cells were more proliferating than DU145 cells, and the reintroduction of PTEN in PC3 cells by transfection (PTEN-PC3) reduced cell proliferation, as shown in Fig. 2C and in agreement with several data-sets obtained with other PTEN-transfected cells. Moreover, EGF increased DU145 cell proliferation strongly.

Figure 2  Akt activation in DU145 (A) and PC3 (B) cells by 10% serum and/or by 50 ng/ml EGF. PTEN transfection reduces growth of PC3 cells in the basal condition (C) but not in the presence of 50 ng/ml EGF (D) as shown also in DU145 cell line; LY295002 (E) and gefitinib (F) reduce dose-dependently the EGF-mediated p-Akt activity in DU145 cells. We treated PCa cells with 50 ng/ml EGF for 30 min in the presence or absence of 10% serum and verified PI3K activity by evaluating phosphorylation of Akt in Western blotting, loading 40 μg protein cell extract/lane. Filters were stripped and blotted with an antibody against total Akt. Akt activation is modulated after EGF treatment only in DU145 cells whereas Akt activation is not affected by EGF in PC3 cells possessing elevated basal PI3K activity. Then the effects of LY294002 and getifinib were evaluated in DU145 cells. Tyrosine kinase inhibitors were added 15 min prior to 50 ng/ml EGF treatment. EGF was left 30 min prior to cell-extract preparation. Gefitinib and LY294002 were dissolved in DMSO and used at a final concentration of 0.1%. The results are representative of at least three independent experiments.
(as well as PTEN-PC3 cell proliferation), whereas it only increased cell proliferation weakly in PC3 cells (Fig. 2D).

An inhibitor of PI3K (LY294002) was able to inhibit EGF-mediated p-Akt expression in DU145 cells (Fig. 2E). In contrast, LY294002 inhibited the basal p-Akt expression in PC3 cells (data not shown). Gefitinib was able to reduce EGF-mediated p-Akt expression in DU145 cells (Fig. 2F), suggesting that in these cells the effects of gefitinib were associated with Akt downmodulation.

**Gefitinib was able to reduce PC3 and DU145 cell proliferation**

As shown in Fig. 3, growth of DU145 cells (B) was strongly inhibited by gefitinib in a dose-dependent manner with an IC50 of 0.35 µM when cells were cultured in serum-free medium plus EGF. The gefitinib effect was less in PC3 cells (A), with an IC50 value of 0.70 µM. Serum was able to reduce the effects of gefitinib significantly. Under these conditions the IC50 values for gefitinib in both cell lines were similar (0.55 µM for DU145 and 0.75 µM for PC3 cells). The growth-curve analyses were in agreement with results from [3H]thymidine uptake (Fig. 3C) in these cells.

**LY294002 treatment sensitizes PTEN-negative cells to gefitinib antiproliferative and pro-apoptotic actions**

PC3, DU145 and PTEN-transfected PC3 cells were tested for proliferation and apoptosis after treatment with two structurally distinct and specific PI3K inhibitors, wortmannin and LY294002. To elucidate the role of PI3K in regulating proliferation, cells were seeded and cultured for 24 h, followed by incubation in the presence or absence of PI3K inhibitors and cell number, [3H]thymidine uptake and the cell cycle at 24, 48 and 72 h of incubation were analysed. PI3K inhibition with 30 µM LY294002 induced relatively lower basal inhibitory effects in DU145 and PTEN-transfected PC3 cells when compared with wild-type PC3 cells (Fig. 4A). Antiproliferative effects induced by LY294002 were attenuated by EGF, especially in PC3 cells. In fact, in the presence of EGF the growth of DU145 cells was significantly inhibited by LY294002 treatment whereas weaker effects were observed in PC3 cells (Fig. 4B).

Thus Erb-B1 activation is able to reduce the antiproliferative effect of PI3K inhibitors only in PC3 cells. Therefore dual inhibition of EGFR and PI3K activities may theoretically be additive. Next we performed combined experiments in PTEN-positive and PTEN-negative cells, as shown in Fig. 5. Growth curves (Fig. 5A and B) and [3H]thymidine uptake (Fig. 5C) indicated that LY294002 possesses additive effects in PTEN-positive cells and synergistic effects in PTEN-null cells.

**PI3K regulates G1 cell-cycle progression in PCa cells**

To determine whether the inhibition of PI3K activity by LY294002 affects cell-cycle progression, DU145, PC3 and PTEN-PC3 cells were treated as described above and cell-cycle distribution was analysed by flow cytometry. We observed that LY294002 determines a
G₀/G₁ arrest, especially in PTEN-null PC3 cells followed by a strong cell apoptosis, evident after 48 h, in a dose-dependent manner. Serum presence and EGF partially counteracted apoptotic events in PC3 cells. LY294002 induced apoptosis strongly after EGF treatment in DU145 and PTEN-PC3 cells, as shown in Table 2. In Fig. 6 we show the FACS profiles in PC3 cells treated with 10 μM LY 294002 and 1 μM gefitinib alone or in combination. The numerical data are summarized in Table 3 whereas Table 4 shows the dual blockade of EGFR and PI3K phosphorylation effects in the eight PCa cell lines tested.

**Dual blockade of EGFR and MAPK activity potentiates growth inhibition of PCa cells in PTEN-positive cells**

Since inhibition of the PI3K/Akt pathway appears to be important but not sufficient for gefitinib-mediated growth inhibition, we decided to study the effects of gefitinib on MAPK pathways. The ERK/MAPK pathway is a prominent signalling pathway activated by receptor tyrosine kinases, including Erb-B1. PC3, DU145 and PTEN-transfected PC3 cells were tested for proliferation and apoptosis after treatment with two MAPK inhibitors, PD98059 and U0126. First we observed that EGF induced a dose-dependent ERK activation; this effect was abolished by MAPK pathway inhibitors (50 μM PD98059 and U0126) and by gefitinib in all cell lines. PD98059 is a potent, selective and cell-permeable inhibitor of MAPK kinase and inhibits phosphorylation of MAPK. U0126 is a potent and selective MEK1/2 inhibitor. In Fig. 7A we show
ERK activity in PC3 cells. Similar results were obtained with DU145 and PTEN-PC3 cells (data not shown). To elucidate the role of MAPK in regulating the proliferation, cells were seeded and cultured for 24 h, followed by incubation in the presence or absence of MAPK inhibitors and the cell number, [3H]thymidine uptake and the cell cycle at 24, 48, and 72 h of incubation were analysed. In Fig. 7C we show the effects of PD98059 and U0126 on [3H]thymidine uptake evaluated after 48 h of culture.

To determine whether proliferative signals can be transmitted through the ERK/MAPK pathway, proliferation was quantitatively evaluated in the presence of PD98059 as percentage of the control. In Table 5 we show the additive (A) and synergistic (S) antiproliferative effects of PI-3K and EGFR dual blockade in the eight PCa cell lines tested. Apoptosis was also measured, and data are shown in Table 6.

Taken together, the data illustrated in this section indicate that although signalling through the ERK/MAPK pathway is not obligatory for cell survival, cytoprotective signals can be transmitted through this pathway in response to Erb-B1 activation and that MAPK effectively influences cell proliferation.

**Discussion and conclusion**

In this study we have identified the PI3K pathway as an essential mediator of cell survival and the MAPK pathway as an essential mediator of constitutive and

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Basal</th>
<th>Serum (10%)</th>
<th>EGF (50 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>&lt;5</td>
<td>53±7</td>
<td>&lt;5</td>
</tr>
<tr>
<td>PC3-PTEN</td>
<td>&lt;5</td>
<td>7±2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>DU145</td>
<td>&lt;5</td>
<td>10±2</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

**Figure 6** Comparison between PC3 and DU145 cells of LY294002-induced apoptosis under basal conditions. Combined effects of 5 µM LY294002 and 1 µM gefitinib in PC3 cells are also shown (bottom panels). Cells (1×10⁶) were harvested after 48 h of treatment with 10 µM LY294002 and analysed for apoptosis by FACS as described in Materials and methods section. SFM, serum-free medium.
Apoptosis was measured as percentage of apoptotic cells. 

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Baseline</th>
<th>Gefitinib (1 μM)</th>
<th>LY 10 μM</th>
<th>Gefitinib + LY294002</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>&lt;5</td>
<td>23 ± 4</td>
<td>35 ± 7</td>
<td>79 ± 10</td>
</tr>
<tr>
<td>PC3-PTEN</td>
<td>&lt;5</td>
<td>32 ± 2</td>
<td>10 ± 4</td>
<td>75 ± 7</td>
</tr>
<tr>
<td>DU145</td>
<td>&lt;5</td>
<td>40 ± 6</td>
<td>15 ± 3</td>
<td>70 ± 13</td>
</tr>
</tbody>
</table>

Growth factor-activated cell proliferation in PCa cells in vitro. Indeed, attenuation of signalling through PI3K inhibitors triggered a rapid and extensive apoptotic response whereas the inhibition of the MAPK pathway determined primarily a significant reduction of cell proliferation.

PI3K and its downstream effectors can be activated constitutively (PTEN-negative cells) or ligand-activated (PTEN-positive cells) for multiple receptor tyrosine kinases, including receptors for several polypeptide growth factors. MAPK and its downstream effectors can be activated by growth factors including the members of EGFR family through the Ras/Raf/MAPK pathway (Barnes et al. 2003) and enhanced MAPK activity induces survival in malignant PCa cells (Zelivinski et al. 2003, Uzgare & Isaacs 2004). Importantly, however, analysis of PI3K and Akt kinase activities (after LY294002 and EGF treatment) indicated that survival signals stimulated by Erb-B1 ligands are also likely to be transmitted through a PI3K-independent pathway. This was demonstrated by experiments in which the protective effect of Erb-B1 activation by ligand was abolished when LY294002 and PD98059 were used in combination (Knowlden et al. 2003, She et al. 2003, Jelovac et al. 2005). This finding indicates that Erb-B1-activated survival signals can also be transmitted through the ERK/MAPK pathway, despite the fact that the MEK inhibitor, when used alone, did not induce apoptosis.

In parallel to the current results, several recent reports suggest that constitutively active MAPK and Akt may contribute to resistance to EGFR inhibitors. Investigating a panel of tumour cell lines, Janmaat et al. (2003) found that EGFR inhibitors induced growth inhibition in A431 cells but not in a series of lung cancer cell lines. Further, persistent activity of either MAPK or PI3K/Akt pathways was observed in resistant lung cancer lines. Using MDA-468 breast cancer cells, Ciardiello et al. (2000) and She et al. (2003) showed that resistance to gefitinib was associated with loss of PTEN and consequent over-activation of Akt with uncoupling of the Akt pathway from EGFR. Reconstitution of PTEN in these cells re-established EGFR-driven Akt signalling and thereby restored gefitinib sensitivity. These results suggest that MAPK and/or Akt signalling pathways may play a central role in the development of resistance to EGFR inhibitors. To study the effects of gefitinib in PCa cell lines, we analysed the signalling-pathway molecules p42/44 MAPK and Akt, which are activated by Erb-B receptors and play an important role in receptor-mediated proliferation and apoptosis, as

Table 3 Effects of gefitinib and LY294002 (L4) alone or in combination on apoptosis of PC3, PTEN-PC3 and DU145 cells. Similar differences were observed for other PTEN-negative or -positive cell lines. Apoptotic cells were detected by a quantifiable peak in sub-G1 phase corresponding to the red fluorescence emitted by a sub-diploid nuclei of cells, and the result were expressed as the cell death percentage present in the sample.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ly294002 (10 μM)</th>
<th>Gefitinib (0.1 μM)</th>
<th>Ly294002 + Gefitinib</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>25.4 ± 5.0</td>
<td>7.5 ± 2.1</td>
<td>55.0 ± 10.5</td>
<td>S</td>
</tr>
<tr>
<td>PC3-PTEN</td>
<td>4.0 ± 1.5</td>
<td>16.0 ± 4.5</td>
<td>19.6 ± 6.0</td>
<td>A</td>
</tr>
<tr>
<td>ALVA31</td>
<td>25.0 ± 4.5</td>
<td>5.0 ± 3.0</td>
<td>60.5 ± 12.5</td>
<td>S</td>
</tr>
<tr>
<td>TSU-Pr1</td>
<td>28.4 ± 3.5</td>
<td>4.5 ± 2.7</td>
<td>58.9 ± 13.5</td>
<td>S</td>
</tr>
<tr>
<td>DU145</td>
<td>7.0 ± 1.4</td>
<td>24.7 ± 5.5</td>
<td>27.5 ± 3.7</td>
<td>A</td>
</tr>
<tr>
<td>ND1</td>
<td>5.4 ± 1.2</td>
<td>25.3 ± 3.7</td>
<td>30.3 ± 4.5</td>
<td>A</td>
</tr>
<tr>
<td>CWR22R-2152</td>
<td>10.3 ± 3.5</td>
<td>21.4 ± 3.7</td>
<td>45.3 ± 7.5</td>
<td>A</td>
</tr>
<tr>
<td>LnCaP</td>
<td>15.4 ± 5.2</td>
<td>20.5 ± 3.5</td>
<td>65.7 ± 13.5</td>
<td>S</td>
</tr>
</tbody>
</table>

*Mean of LY294002-induced cell apoptosis was 23.5 ± 5.6 (mean ± so) for PTEN-null cells and 6.7 ± 2.7 for PTEN positive cells (P<0.005).

†Mean of gefitinib-induced cell apoptosis was 9.4 ± 3.8 for PTEN-null cells and 21.8 ± 2.1 for PTEN-positive cells (P<0.05).

‡Mean of combination-treatment-induced cell apoptosis was 60.0 ± 2.2 for PTEN-null cells and 30.5 ± 5.5 for PTEN-positive cells (P<0.005).

Apoptosis was measured as percentage of apoptotic cells.
already described. EGF induced phosphorylation of Akt only in PTEN-positive cells and this phosphorylation was completely prevented in the presence of gefitinib, whereas p42/44 MAPK was phosphorylated both in PTEN-negative and -positive cells after EGF treatment. This inhibition of downstream signalling occurred at the same gefitinib concentration that inhibited EGF-induced phosphorylation of EGFR. In addition we compared the effects of gefitinib with PTEN expression and constitutive and activated Akt and ERK activities. We indicated that PTEN is positively related to gefitinib activity and its loss determines a reduced effectiveness of the drug. The basal activity of Akt as well as of ERK are negative mediators of gefitinib effectiveness. High levels of Akt through a downregulation or loss of PTEN cause a reduced sensitivity of cells to gefitinib treatment. Thus combined treatments with pharmacological inhibitors of PI3K are highly synergistic in these cells. PTEN-positive cells, which possess very low levels of Akt, are

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Additive (A) and synergistic (S) antiproliferative effects of MAPK and EGFR dual blockade. Antiproliferative effects were measured as % of inhibition vs control.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>PD98059 (30 μM)*</td>
</tr>
<tr>
<td>PC3</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>PC3-PTEN</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>ALVA31</td>
<td>15.0 ± 0.3</td>
</tr>
<tr>
<td>TSU-Pr1</td>
<td>15.6 ± 3.2</td>
</tr>
<tr>
<td>DU145</td>
<td>17.6 ± 2.7</td>
</tr>
<tr>
<td>ND1</td>
<td>20.1 ± 2.5</td>
</tr>
<tr>
<td>CWR22R-V15</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>LnCaP</td>
<td>5.4 ± 0.7</td>
</tr>
</tbody>
</table>

*Mean of PD98059-induced cell-growth inhibition (% vs control) was 9.8% ± 3.2% (mean ± so) for PTEN-null cells and 11.5% ± 4.3% for PTEN-positive cells (P=0.756). This parameter was significantly co-related to p-Erk basal activity shown in Table 1 with r=0.708 and P=0.049.
†Mean of gefitinib-induced cell-growth inhibition (% vs control) was 14.1% ± 3.8% for PTEN-null cells and 31.8% ± 1.9% for PTEN-positive cells (P<0.05).
‡Mean of combination-treatment-induced cell-growth inhibition (% vs control) was 31.1% ± 9.2% for PTEN-null cells and 46.7% ± 11.8% for PTEN-positive cells (P=0.337).
Apoptosis was measured as percentage of apoptotic cells. The result were expressed as the cell death percentage present in the sample. Additive (A), synergistic (S) or null (N) pro-apoptotic effects of MAPK and EGFR dual blockade. Apoptotic cells were detected by a quantifiable peak in sub-G1 phase corresponding to the red fluorescence emitted by a sub-diploid nuclei of cells, and the result were expressed as the cell death percentage present in the sample.

Table 6 Additive (A), synergistic (S) or null (N) pro-apoptotic effects of MAPK and EGFR dual blockade. Apoptotic cells were detected by a quantifiable peak in sub-G1 phase corresponding to the red fluorescence emitted by a sub-diploid nuclei of cells, and the result were expressed as the cell death percentage present in the sample.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PD98059 (30 μM)*</th>
<th>Gefitinib (0.1 μM)†</th>
<th>PD + Gefitinib‡</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>0.8</td>
<td>7.5 ± 2.1</td>
<td>10.0 ± 10.5</td>
<td>N</td>
</tr>
<tr>
<td>PC3-PTEN</td>
<td>0.8</td>
<td>16.0 ± 4.5</td>
<td>19.0 ± 6.0</td>
<td>N</td>
</tr>
<tr>
<td>ALVA31</td>
<td>0.8</td>
<td>10.0 ± 3.0</td>
<td>10.5 ± 2.5</td>
<td>N</td>
</tr>
<tr>
<td>TSU-Pr1</td>
<td>10.4 ± 3.5</td>
<td>10.3 ± 2.7</td>
<td>48.9 ± 13.5</td>
<td>S</td>
</tr>
<tr>
<td>DU145</td>
<td>11.0 ± 1.4</td>
<td>24.7 ± 5.5</td>
<td>47.5 ± 3.7</td>
<td>S</td>
</tr>
<tr>
<td>ND1</td>
<td>111.4 ± 1.2</td>
<td>25.3 ± 3.7</td>
<td>30.3 ± 3.6</td>
<td>A</td>
</tr>
<tr>
<td>CWR22R-2152</td>
<td>8.3 ± 3.5</td>
<td>21.4 ± 3.7</td>
<td>27.3 ± 4.7</td>
<td>A</td>
</tr>
<tr>
<td>LnCaP</td>
<td>5.5</td>
<td>27.5 ± 3.5</td>
<td>30.7 ± 5.5</td>
<td>A</td>
</tr>
</tbody>
</table>

*Mean of PD98059-induced cell apoptosis was 4.4 ± 2.3 (mean ± so) for PTEN-null cells and 7.9 ± 2.5 for PTEN-positive cells (P=0.342). This parameter was not related with p-Erk basal activity shown in Table 1 with r=0.354 and P=0.390.
†Mean of gefitinib-induced cell apoptosis was 9.4 ± 3.8 for PTEN-null cells and 21.8 ± 2.1 for PTEN-positive cells (P<0.05).
‡Mean of combination-treatment-induced cell apoptosis was 25.0 ± 9.3 for PTEN-null cells and 31.0 ± 6.0 for PTEN-positive cells (P=0.607).

Apoptosis was measured as percentage of apoptotic cells.

stimulated in this way by EGFR triggering. In fact, EGF induces Akt activation, which is reduced by gefitinib treatment. In these cells the combination with PI3K inhibitors determines only very weak additive effects. Similarly, high basal levels of ERK activity cause a reduced sensitivity of cells to gefitinib treatment. In this case, the combined treatments with pharmacological inhibitors of MAPK are highly synergistic in cells possessing elevated p-ERK levels (TSU-Pr1, DU145 and ND1 cells) independently to PTEN status. As previously described, gefitinib effectiveness was independent of EGFR levels whereas an increased expression of Her2 was a negative parameter for predicting gefitinib effectiveness. This is in agreement with our observation in primary cultures of PCa treated with an anti-androgen receptor such as Casodex, in which increased expression of Her2 with decreased expression of PTEN inhibit the effects of gefitinib (C Festuccia, P Muzi, D Millimaggi, L Biordi, G L Gravina, S Speca, A Angelucci, V Dolo, C Vicentini, M Bologna, unpublished work) or with the observations in vivo in CWR22 relapsed xenografts after long-term treatment with gefitinib in which the drug resistance seems to be associated with Her2 increment.

Taken together, our results indicate that gefitinib treatment could be used as a monotherapy only in PCa cells that express PTEN protein and possess low basal p-ERK levels. Nevertheless, it is also possible that these selected cell populations can present de novo resistance to gefitinib after prolonged treatment with the drug, through an increased activity of ERK due to the increment of other growth factor receptors GFR including insulin-like growth factor I receptor (IGF-IR; Yamakoa et al. 2002, Jones et al. 2004, Camirand et al. 2005) or the Trk family, as some unpublished data of ours indicate. Therefore, gefitinib could be conveniently used in combination treatment schedules with (1) PI3K/Akt pathway inhibitors (for example with the pharmacologically active PI3K inhibitor, perifosine (Van Ummersen et al. 2004, Vink et al. 2005) or with mammalian target of rapamycin (mTOR) inhibitors (for reviews see Goudar et al. 2005, Vignot et al. 2005), (2) Ras/Raf/MAPK-pathway inhibitors or (3) IGF-IR inhibitors, which can be useful to slow down the onset of drug-resistant cells and possibly to control the progression and the proliferation in advanced PCas.

Acknowledgements

Gefitinib is a trademark of the AstraZeneca group of companies. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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