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Targeting the EGF receptor in ovarian cancer with the tyrosine kinase inhibitor ZD 1839 (‘Iressa’)
MATERIALS AND METHODS

Cell lines

The human ovarian carcinoma cell lines used were: PE01 and PE04 (Langdon et al., 1988), SKOV-3 and A2780 (European Collection of Animal Cell Cultures, Porton Down, UK), and OVCAR-5 (kindly provided by Dr T Hamilton, Fox Chase Institute, PA, USA). All lines were routinely cultured at 37°C in a humidified atmosphere of 5% CO₂ in air, using ‘full media’; RPMI 1640 containing phenol red (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum (FCS), t-glutamine (2 mM), penicillin (100 IU ml⁻¹) and streptomycin (100 µg ml⁻¹).

Growth assays

Log-phase cells were trypsinized and seeded in full media into 24-well plates (Falcon) in quadruplicate at densities between 0.5–2.5 × 10⁵ cells per well⁻¹. After 24 h media was replaced (following two phosphate-buffered saline (PBS) washes) with phenol red free RPMI 1640 (PRF-RPMI) containing 5% dextrancoated stripped foetal calf serum, penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹), glutamine (2 mM). After a further 24 h, drugs and fresh media were added to cells in PRF-RPMI 1640; designated day 0. Tyrosine kinase inhibitor ZD 1839 was added 30 min prior to the addition of TGFβ (1 nM). Drugs and media were replaced on day 2, then cells harvested on day 5 and counted using a Coulter counter (Coulter Electronics Ltd., Luton, UK).

Western blotting

Cell lines were seeded in full media into 60 cm² petri dishes (Nunclon). After 24 h, cells were washed twice with PBS before adding PRF-RPMI. A further 24 h later, (cells 60–80% confluent) plates were treated for 30 min with PRF-RPMI media in the presence or absence of ZD 1839 (0.03–3.0 µM). Media ± ZD 1839 was then replenished and incubated for a further 15 min in the presence or absence of TGFβ (1 nM). Cells were washed twice in ice cold PBS, and lysed using 1 ml hypotonic lysis buffer (50 mM Tris-HCl (pH 7.5), 5 mM EGTA (pH 8.5), 150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 20 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride, 10 µg ml⁻¹ leupeptin, 10 µg ml⁻¹ aprotinin and 10 mM sodium molybdate). Lysates were clarified by centrifugation for 6 min at 13 000 r.p.m. in a microtube at 4°C. Total protein concentrations of supernatants were determined using a Bio-rad protein Assay Kit (Bio-rad, Richmond, CA, USA).

Samples (35–75 µg) were denatured at 95°C for 5 min in buffer containing SDS and mercaptoethanol and resolved by 7.5% SDS – PAGE. They were transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA) overnight at 4°C. After transfer, membranes were blocked with 1% blocking reagent (containing 10% purified casein protein in maleic acid, Boehringer Mannheim) diluted in TBS (Tris buffered saline; 20 mM Tris-HCl, 137 mM NaCl, pH 7.5) for 1 h at room temperature. Primary antibodies were added in 0.5% TBS/blocking solution and left overnight at 4°C: EGFR (Neomarkers Ab12) 1/200, phosphotyrosine (Santa Cruz P2Y0) 1/200, phospho-ERK (NEB 9101) 1/1000 and phospho-AKT S473 (NEB 9271) 1/1000. Membranes were then washed; 3 × 5 min TBS-T (TBS with 0.1% Tween 20), and 2 × 5 min in 0.5% TBS/blocking solution, then incubated with appropriate secondary antibody for 1 h at room temperature. Membranes were washed again; 3 × 5 min with TBS-T, 3 × 5 min with TBS. After 1 min incubation in room temperature luminescence substrate solution, light emission was detected on X-ray film.

RT – PCR

Total cellular RNA was extracted from cells in log phase growth using TRI reagent™ (Sigma, Poole, UK). Samples were treated with 20 U/50 µl DNase 1 (Boehringer Mannheim, East Sussex, UK) to remove genomic DNA contamination. RNA was then re-extracted using a phenol/chloroform protocol. Reverse transcription was performed with a first strand cDNA Synthesis kit (Boehringer Mannheim) using the oligo dT primer provided. RNA (1 µg) yielded 20 µl of cDNA of which 2 µl was used for each subsequent PCR reaction with each primer pair (Table 1). PCR reactions (20 µl) contained: 1× buffer, 1.5 mM MgCl₂, 0.2 mM dNTP mixture, 1.0 U Taq polymerase (ICRF, Clare Hall, South Mimms, UK), 500 nM each primer (ICRF). RNA which had not been reverse transcribed was used in PCR reactions to check for genomic contamination of RNA samples.

Xenograft experiments

The PE04 xenograft was initiated from the cultured PE04 ovarian cancer cell line and maintained subcutaneously in the flanks of female nude mice. Animals were at least 8 weeks old at the time of experimentation and were maintained in negative pressure isolators (La Calhene, UK). For experiments, tumour fragments were implanted and allowed to develop to 4–6 mm (period of approximately 1 month). Animals were then allocated to treatment (five mice per group) or control (10 mice per group) groups and treatment commenced (defined as day 0).

Treatment groups contained 8–10 tumours and control groups 14–18 tumours. The drug was given orally as a suspension in 0.5% polyoxymethyleneorbitan (Tween 80) for 14 days. Tumour size was measured twice weekly using calipers and the volume calculated according to the formula: V=6×length × width². Relative tumour volumes were then calculated for each individual tumour by dividing the tumour volume on day t (Vt) by the tumour volume on day 0 (V₀) and multiplying by 100. Body weight changes were monitored every 4 days to monitor toxicity. All experiments were conducted according to UKCCCR (1998) guidelines under a Home Office licence.

Statistical analysis

The Student t-test was used to compare differences between groups.

RESULTS

The tyrosine kinase inhibitor ZD 1839 inhibits TGFβ stimulated growth of ovarian cancer cells in vitro

Five ovarian cancer cell lines (PE01, PE04, SKOV-3, OVCAR-5 and A2780) were investigated for their response to the EGFR-targeted

<table>
<thead>
<tr>
<th>Table 1</th>
<th>PCRprimers used</th>
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<tr>
<td><strong>PCR primers</strong></td>
<td><strong>Product size (bp)</strong></td>
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<tr>
<td><strong>EGFR</strong></td>
<td><strong>Antisense</strong> S⁵-CACATCTCCATCACATTATCTCC-3' 516</td>
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<tr>
<td></td>
<td><strong>Sense</strong> S⁵-ACCTCCGTTAAGAGTGTCC-3' 474</td>
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<tr>
<td><strong>γ-Actin</strong></td>
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<tr>
<td></td>
<td><strong>Sense</strong> S⁵-CAAGTTCTACAATCCAGTGC-3' 316</td>
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<tr>
<td><strong>TGF-α</strong></td>
<td><strong>Antisense</strong> S⁵-CCGCTCTGGCTA TTGTTGTGG-3' 474</td>
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<td><strong>Sense</strong> S⁵-GGTCCGCTGA TTTCCTCCTC-3' 316</td>
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<tr>
<td><strong>EGF</strong></td>
<td><strong>Antisense</strong> S⁵-AGGAAGATGAGCCAACATTACTCC-3' 397</td>
</tr>
<tr>
<td></td>
<td><strong>Sense</strong> S⁵-TTCTTTTGTTCTCCAGTCTGTC-3' 397</td>
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Inhibition of tyrosine phosphorylation of the EGFR by ZD 1839

The cell lines were pre-treated with ZD 1839 for 30 min before a 15 min incubation in the presence or absence of 1 nM TGFz. TGFz increased tyrosine phosphorylation of both the EGFR and ErbB2 in the four responsive cell lines (PE01, PE04, SKOV-3 and OVCAR-5), consistent with ligand activation via the EGFR and heterodimerization with ErbB2 (Figure 4A). A2780 showed no band for either EGFR or ErbB2. The identity of bands had been confirmed in previous experiments using antibodies specific for the EGFR and erbB2 (data not shown). At concentrations of ZD 1839 > 0.3 μM, the EGFR phosphorylation was completely blocked in all cell lines except OVCAR-5 in which EGFR phosphorylation was blocked at concentrations of ZD 1839 > 1 μM. The ErbB2 phosphorylation was also decreased but to a lesser degree in all four responsive lines (Figure 4A). In the absence of TGFz, only ErbB2 phosphorylation was observed in untreated cells (Figure 4A). This was also decreased after addition of ZD 1839 but only at higher concentrations of inhibitor.

Inhibition of the ERK and PI3-kinase pathways by ZD 1839

Lysates of the five cell lines were also probed with antibodies recognizing phospho-ERK, a member of the MAP kinase cascade, and to phospho-AKT ([2], downstream of PI-3 kinase. TGFz increased phosphorylation of both ERK 1 and 2 (p44 and p42) in the four responsive cell lines, but not in A2780 (Figure 4B). ZD 1839 blocked ERK phosphorylation at 1 and 3 μM, reflecting the results for EGFR and erbB2 tyrosine phosphorylation (Figure 4A). A similar result was obtained for phospho-AKT, with the four responsive lines showing phosphorylation induced by TGFz, which was blocked in all but the SKOV-3 cell line at the lower concentration of 0.3 μM ZD 1839. Both phospho-ERK and phospho-AKT were elevated at basal conditions in A2780 cells compared to the other cell lines and neither TGFz nor ZD 1839 had any effect on expression (Figure 4B,C).

Inhibition of the PE04 ovarian cancer xenograft by ZD 1839

The ability of ZD 1839 to inhibit growth in vivo was assessed using the PE04 ovarian cancer model. Previous xenograft studies have used ZD 1839 at doses of 150–250 mg kg⁻¹ day⁻¹ (Ciardiello et al., 2000; Sirotnak et al., 2000). ZD 1839 when given orally at 200 mg kg⁻¹ day⁻¹ for 14 days produced > 50% inhibition of control growth at the end of the treatment period, and this effect persisted for a further 31 days, at which point the experiment...
was terminated (Figure 5). A lower dose, 50 mg kg$^{-1}$ day$^{-1}$ produced a small effect with statistical significance at day 17 (i.e. immediately after the full treatment course) but this effect did not persist (Figure 5). The effect of continued treatment at either dose was not evaluated. At 50 mg kg$^{-1}$ day$^{-1}$ there was no mean body weight loss compared to control at end of treatment (day 14) and after a further 14 days (day 28) while at 200 mg kg$^{-1}$ day$^{-1}$ there was a small mean weight loss (9.8±1.6% on day 14 and 10.9±4.5% on day 28).

**DISCUSSION**

Strategies targeting the EGFR may well have therapeutic potential in ovarian cancer as the receptor is frequently overexpressed in this disease and patients with tumours expressing high levels of EGFR tend to have a poor prognosis. ZD 1839 is one of the latest inhibitors developed against this receptor and, in addition to being both potent and selective, it is orally active. This is the first detailed study of ZD 1839 in a range of ovarian cancer cell lines and our results extend the previous observations of broad spectrum activity for this agent against a range of xenograft types including cancers of the lung, colon, prostate and vulva (Ciardiello et al., 2000; Sirotnak et al., 2000). We observed good reversal of EGFR tyrosine phosphorylation produced by TGF-α activation in ovarian cancer cell lines and this was consistent with growth inhibition at these concentrations. In the absence of added TGF-α, the cell lines were shown to produce mRNAs for several EGFR-activating ligands indicating the strong possibility of autocrine regulation. Under these conditions, ZD 1839 partially inhibited growth of several of the cell lines consistent with blockade of activation via this endogenous expression. Extension into a xenograft model confirmed the activity of the drug given orally to the PE04 ovarian cancer in vivo. We have previously investigated the activity of another anilinoquinazoline, ZM 252868 (PD 153035) which had similar in vitro properties to those of ZD 1839; however its activity in vivo was limited and while it demonstrated transient reduction of EGFR tyrosine phosphorylation in A431 xenografts, this was insufficient to produce in vivo growth inhibition (Kunkel et al., 1996). ZD

![Image](image-url)
Figure 4 Western blot of ZD 1839 treated cell lines using (A) the anti-phosphotyrosine PY20 antibody, (B) the phospho-ERK NEB 9101 antibody and (C) the phospho-AKT NEB 9271 antibody. Blots are shown for the PE01, PE04, SKOV-3, OVCAR-5 and A2780 cell lines. ZD 1839 was added 30 min prior to TGFα (1 nM) addition and lysates were collected after 15 min exposure to TGFα. Amounts of protein loaded were 75 µg lane⁻¹ for the phosphotyrosine gel and 35 µg for the phospho-ERK and phospho-AKT gels. Exposure times varied from 20 s to 2 min but were the same for each antibody.

Figure 5 Effect of ZD 1839 on the growth of established PE04 xenograft grown in nude mice. Groups of nude mice received either vehicle only or daily oral treatment of ZD 1839 at either 200 mg kg⁻¹ day⁻¹ or 50 mg kg⁻¹ day⁻¹. Points shown are mean values for groups of 5–8 tumours. Error bars= standard deviation. Statistically significant differences from control: *P<0.05, Student t-test.
1839 possesses significant in vivo activity and is now being evaluated in clinical trials.

Western blot probing for tyrosine phosphorylation confirmed that ZD 1839 was acting as predicted, with the inhibitor targeting the EGFR and having a secondary effect on ErbB2 phosphorylation. The latter is likely to be due to inhibition of heterodimer phosphorylation. Furthermore, the same concentrations of ZD 1839 shown to inhibit receptor activation were associated with growth inhibition. Phosphorylation events in the downstream effector molecules of the MAP kinase (phospho-ERK) and PI-3 kinase (phospho-AKT) pathways revealed some of the complexity involved with signal transduction from these receptors. There was a striking similarity between phosphorylation and hence activation of the ErbB receptors in response to both TGFβ and EGF. It is apparent from these data that levels of EGFR protein expression are not simply associated with magnitude of ligand response as even cell lines with low levels of EGF can show a marked stimulation when treated with TGFβ. However, tyrosine phosphorylation of the EGFR in clinical specimens of ovarian cancer can be readily identified and it seems likely that those tumours in which activation is found represent the target population for these agents.

In conclusion, these data indicate that the EGFR-targeted tyrosine kinase inhibitor ZD 1839 can inhibit growth of ovarian cancer cells in vitro and in vivo, consistent with inhibition of tyrosine phosphorylation at the EGFR. These results lend further support to the view that targeting the EGFR in ovarian cancer could have therapeutic value.

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References


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Targeting the EGFR receptor
JM Sewell et al

Experimental Therapeutics


