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Receptor tyrosine kinase (RTK) inhibition is effective in chemosensitising EGFR-expressing drug resistant human ovarian cancer cell lines when used in combination with cytotoxic agents

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ABSTRACT

This study has focused on the use of RTK inhibitors in the treatment of ovarian cancer. We have used the human ovarian cancer cell line PEO1 alongside two in-house derived drug resistant variants: PEO1CarboR (8-fold acquired resistance to carboplatin and cisplatin) and the Pgp expressing PEO1TaxR (15-fold acquired resistance to paclitaxel). These variant cell lines were shown to have a higher expression of EGFR 1.6- and 2.0-fold increase, respectively, compared with the parental cell line.

We have shown that the RTK inhibitor GW282974A (an analogue of GW2016; lapatinib) is effective in chemosensitisation of drug resistant EGFR over-expressing cells giving rise to a synergistic effect when used in combination with either cisplatin or paclitaxel in chemosensitivity assays. These effects were also seen at the level of apoptosis using the Annexin V assay and expression levels of the IAP Survivin. A reduction in the downstream signalling effector phosphorylated ERK was seen in both resistant cell lines when GW282974A was used in combination with either cisplatin or paclitaxel. This reduction was not so apparent in cells treated with the single agent GW282974A or cytotoxic agent. Interestingly, we did not show evidence for an enhanced sensitivity to the RTK inhibitor in our EGFR expressing resistant lines *versus* parental PEO1 cells. However, the paclitaxel resistant cell line appeared more sensitive to the chemosensitising effects of GW282974A, in line with its increased EGFR expression. Our data suggest that RTK inhibition is effective in circumvention of tumour cell drug resistance that occurs in conjunction with EGFR overexpression.

1. Introduction

Over the last decade, many advances have been made in the understanding of the molecular biology of cancer cells. Growth factor receptor overexpression is a common feature of malignant cells and this is often accompanied by an elevated production of growth factors produced in either a paracrine or autocrine fashion, driving the proliferation of the abnormal

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cells. Aberrant cell cycle regulation and signal transduction pathways can mediate many important events including growth-factor induced proliferation and can also influence the apoptotic sensitivity of tumour cells. Ovarian cancer frequently responds to first line therapy using cytotoxic drugs such as the taxanes and platinum agents. However, drug resistance is a frequent problem and 5 years survival rates are between 20% and 30% at best. Normal ovarian epithelial cells express relatively low levels of epidermal growth factor (EGFR; HER1) whereas, approximately 70% of ovarian cancers express elevated levels of EGFR [1]. Moreover, a number of studies have indicated that overexpression of EGFR is associated with a poor prognosis in a variety of human malignancies, including ovarian cancer [2,3]. There is also evidence of an association of EGFR expression with VEGF expression in ovarian cancers [4].

The idea of using chemotherapeutic agents combined with agents designed to target tyrosine kinase receptor phosphorylation has been explored in a number of laboratory-based studies. For example, Pegram et al. [5] used a c-ERBb2-directed monoclonal antibody with a variety of chemotherapeutic agents including doxorubicin, VP-16, paclitaxel and 5-FU in human tumour xenograft models of a *c-ERBb2*-transfected human breast carcinoma. The effects of the antibody therapy with VP-16 were synergistic and with doxorubicin an additive effect was demonstrated. The dual EGFR and c-ERBb2 directed small molecule tyrosine kinase inhibitor CI1033 used in combination with the topoisomerase-I directed compound SN-38 was shown to be synergistic in T98G glioblastoma cells [6]. Importantly, these effects have translated to clinical trials where the response rate of paclitaxel was significantly enhanced by the use of trastuzumab (Herceptin®), which targets HER2/c-ERBb2 in breast cancer patients (reviewed in [7]).

The small molecule inhibitor lapatinib (GW2016), a specific dual tyrosine kinase inhibitor of EGFR and ErbB2 has shown activity against human tumour xenografts expressing these receptors and also to AKT-overexpressing human tumour xenografts [8]. In addition, a report by Sewell et al. [9] showed a TGFα stimulated growth of PEO1 cells which was reversed by gefitinib (Iressa®), with reduced phospho-ERK1/2 and reduced phospho-AKT expression.

In the present study we have looked at the effects of using a small molecule inhibitor against EGFR in terms of its possible sensitisation effects on drug resistant PEO1 ovarian cancer cells. This has involved use of PEO1CarboR platinum-resistant and PEO1TaxR paclitaxel-resistant ovarian cancer cells treated with combinations of the small molecule inhibitor GW282974A (an analogue of GW2016) and cytotoxic agents. Experiments were designed to assess possible synergistic effects of drug combinations by looking at their effects on chemosensitivity and apoptosis.

2. Materials and methods

2.1. Cell culture

All cell culture reagents were obtained from Sigma (Poole, UK) unless stated otherwise. The ovarian carcinoma parental cell line PEO1 (obtained from Prof. Fran Balkwill, formerly ICRF, London, UK) were cultured as monolayers in RPMI-1640 medium and supplemented with 10% foetal calf serum (FCS, heat inactivated; Invitrogen, Paisley, UK). The drug resistant variants PEO1TaxR and PEO1CarboR were derived by step-wise incubation of the inducing agent over a number of months until a stable resistance phenotype was acquired. This resulted in the PEO1TaxR variant line (maintenance dose 8 nM paclitaxel) with approximately 15-fold resistance, and PEO1CarboR variant line (maintenance dose 2 μM) with approximately 8-fold resistance to both carboplatin and to cisplatin.

2.2. Cytotoxic drugs

Cisplatin (Sigma, Poole, UK) was dissolved as a stock solution in sterile 0.9% saline and stored at −20 °C until use. Paclitaxel was obtained as a pharmacy preparation and diluted in sterile 0.9% saline as a stock solution and stored as for cisplatin. GW282974A (from hereon referred to as GW2974A) was kindly provided by GlaxoWellcome, Durham, NC, USA as a powder and made up as a stock solution in DMSO.

2.3. Receptor-binding assay for EGFR measurement

The PEO1 parental and the two resistant cell lines were plated out in a 96 well plate in duplicate at a density of 5×10^4/well and grown to near confluence. A radioimmunoassay was performed on ice to prevent receptor internalisation (according to the method described in [10]) using the anti-EGFR antibody ICR16 and ^{125}I goat anti rat IgG (Serotec).

2.4. Median effect analysis to look for evidence of synergism with a combination of GW2974A and paclitaxel/cisplatin

Cells were plated in 96 well plates and left for 24 h to attach. Drugs were diluted in supplemented tissue culture medium, added to give the appropriate final drug concentration, with untreated wells being used as controls. The drugs cisplatin, paclitaxel or GW2974A were added singly in step-wise increasing concentrations. The dose ranges used were as follows: cisplatin PEO1 and PEO1CarboR lines 0.25–16 μM; paclitaxel PEO1 0.5–32 nM, PEO1TaxR 1–64 nM. A tray of each cell line was then treated with a combination of the cytotoxic drug and GW2974A over the dose ranges used for the single agent protocol, ensuring that combination of the drugs was always in a fixed ratio. Drugs were present in a fixed ratio for all step-wise concentrations used (e.g. cisplatin:GW2974A was 1:1.25). In wells that contained single drug addition, an appropriate volume of tissue culture medium diluent was added to enable valid comparison of wells that contained combined (dual) drug treatments. In order to avoid significant dilution of the first drug administered to a well, the second drug was added in the smallest volume possible (10 μL in a total well final volume of 260 μL). After 72 h incubation, cell numbers were assessed using the MTT assay. A small number of assays were carried out using methylene blue staining as an alternative read out and data

shown to be in close agreement with the MTT results (data not shown).

2.5. Isobologram analysis

The fraction of cells affected by a particular drug treatment was calculated and used with the Calcusyn programme (Cambridge Biosoft, Cambridge, UK). This software program uses the Combination Index (CI) method described by Chou and Talalay [11], which is based on the multiple drug effect equation. The constant ratio combination approach was used to assess the effect of both drugs in combination, whereby dose–response curves were determined with both drugs in combination, at a fixed ratio that was equivalent to their IC_{50} values. CI values of <1.0 indicate greater than additive effects (synergism where the greater the synergy the smaller the value), CI values equal to 1.0 indicate additivity and CI values >1 indicate antagonism.

2.6. Annexin V apoptosis assay

Cells were seeded into tissue culture flasks to give a density approximately 30% confluence, allowed to attach for 2–3 h and then treated with the appropriate compounds either singly or in combination for a period of 48 h with drug dosing 20 μM GW2974A, paclitaxel 5 nM, cisplatin 5 μM. An Annexin V-FITC conjugated apoptosis detection kit incorporating PI was used as described by the manufacturer's protocol (Oncogene; supplied by CN Biosciences, Beeston, UK). Harvesting of cells involved collecting attached cells following trypsinization and floating cells which were combined in the cell pellet. Samples were analysed by flow cytometry, using the FL1 (FITC) and FL3 (PI) lines and each reading was taken using 10,000 events.

2.7. Western immunoblotting

Exponentially growing cells were treated with either drug alone (cytotoxic agent or GW2974A) or in combination for a total of 48 h for Survivin and ERK analysis using immunoblotting. Whole cell lysates were obtained by trypsinizing the monolayer of adherent cells, combining them with the floating cell population and washing with PBS at 4 °C. Cell pellets were then subjected to osmotic rupture in hypotonic detergent based buffer (1 mM PMSF, $NaVO_4$, aprotinin and leupeptin as protease inhibitors, 150 mM NaCl, in 50 mM Tris buffer, 0.2% SDS, 1% NP-40, pH 7.5) and 40–50 μg of protein/sample electrophoresed on SDS-PAGE gels with subsequent transfer blotting. Membranes were incubated overnight at 4 °C with primary antibody, i.e. to Survivin, ERK2 or to phosphorylated ERK2 (all antibodies were manufactured by Santa Cruz, and obtained from Autogen Bioclear, Calne, UK). After washing, membranes were incubated with a secondary horseradish peroxidase (HRP)-linked appropriate species antibody preparation at room temperature for 1 h with chemiluminescence used for visualization. Following the probing of each membrane with the primary antibody of choice, the membrane was stripped and reprobed using an actin antibody (Santa Cruz; supplied by Autogen Bioclear, Calne, UK) to act as a loading control.

3. Results

3.1. Levels of EGFR measured in PEO1 cell lines

The parental PEO1 cell line showed detectable EGFR levels (in line with the findings of others [12]). Both the PEO1CarboR and PEO1TaxR cells showed increased levels of EGFR expression relative to the parental cells, as indicated in Table 1. We were also able to confirm these results using the same antibody in western immunoblotting (data not shown).

3.2. Effects of combination treatment of GW2974A and cytotoxic drugs in PEO1 cell lines

Fig. 1 shows a selection of typical isobologram graphs obtained showing CI *versus* fractional effect. For the drug resistant cells, all data points are shown to fall below the 1.0 cut-off line and are, therefore, representative of synergism for all the cell lines examined, whether with paclitaxel or with cisplatin in combination with the small molecule inhibitor GW2974A. The adjacent bar graph E shows the reduction in the IC_{50} values obtained for drugs administered in combination *versus* the IC_{50} for the cytotoxic drug added singly (as described in [13]). The effects of the combination for paclitaxel were marked for both parental PEO1 and PEO1TaxR cells, as indicated by the levels of statistical significance for the decrease in IC_{50} value obtained for the cytotoxic drug alone *versus* IC_{50} obtained for the combination with GW2974A. There was a more modest effect seen when cisplatin was used in the combination with PEO1 cells and the effects were largely additive and slightly antagonistic. For PEO1CarboR cells, the effects of chemosensitisation were seen with overall CI values being significantly lower than 1.0. Overall, effects of paclitaxel in combination with GW2974A were the most effective, particularly when seen in paclitaxel resistant cells.

3.3. Effects of combination drug treatment on apoptosis in PEO1 cell lines

Figs. 2 and 3 show the effects of the paclitaxel and GW2974A in the PEO1 and the PEO1TaxR cells, respectively. The extent of apoptosis seen when resistant cells were treated with the combination was close to that seen for the parental cells, whereas the levels of apoptosis for paclitaxel (dose of 5 nM for both cell lines) alone vary significantly for the two cell lines,

Table 1 – Data obtained using receptor-binding assay incorporating radioimmunoassay with monoclonal antibody for EGFR

Cell line	Fold increase over PEO1 parental cells
PEO1	1.00
PEO1CarboR	1.60 (0.24)
PEO1TaxR	2.06 (0.68)

Data shown are taken from three separate experiments carried out with the standard deviation shown in parentheses. All data were obtained as cpm and results are shown compared with a parental cell line result which has been corrected to 1.00 in each experiment.

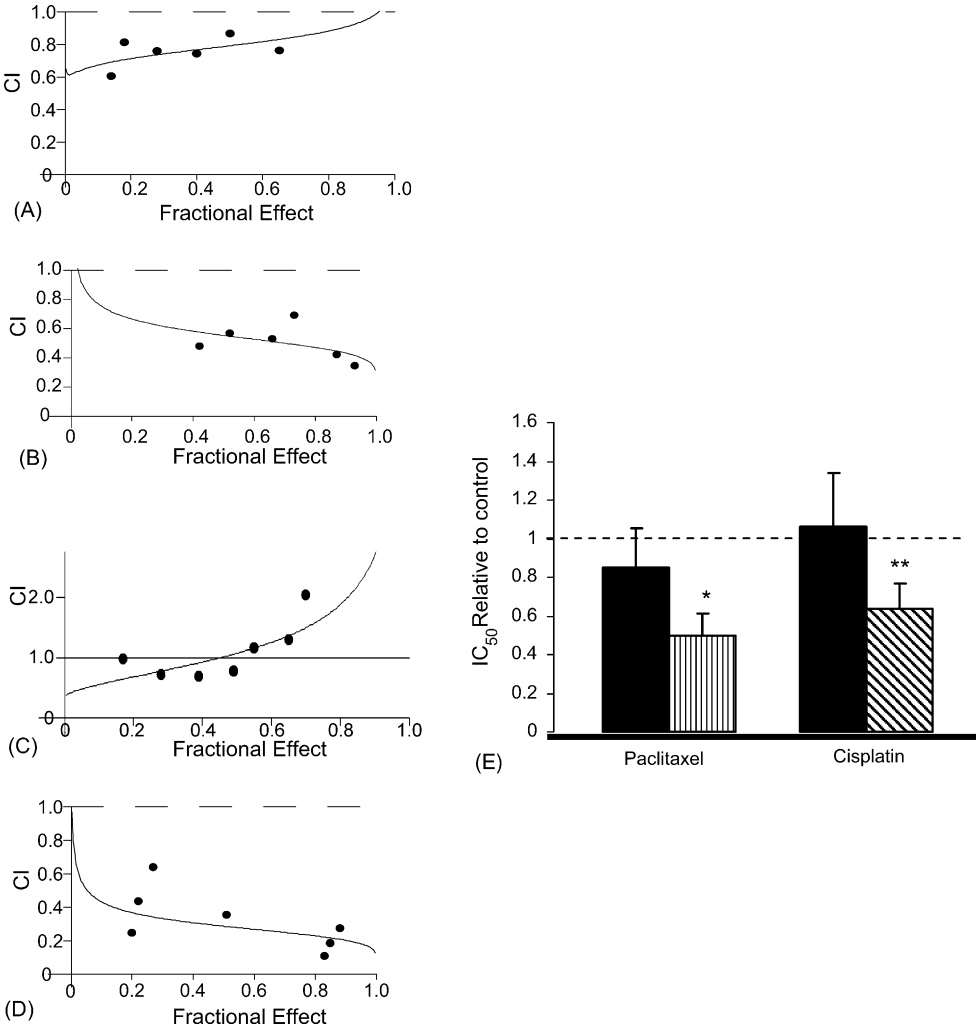

Fig. 1 – Left hand panel shows typical isobologram analyses for: (A) PEO1 with paclitaxel + GW; (B) PEO1TaxR with paclitaxel + GW; (C) PEO1 with cisplatin + GW; (D) PEO1CarboR with cisplatin + GW. Right hand panel shows a bar graph representing the IC_{50} difference relative to control (single cytotoxic drug alone) for PEO1 cells (solid bar), PEO1TaxR (vertical line bar) and for PEO1CarboR (diagonal line bar). Data shown are the means of >3 repeat experiments, with error bars showing the standard deviation. *Indicates *p*-value of 0.023; **indicates *p*-value of 0.006 using the Student's *t*-test.

indicated by the viable cell population levels of 66.0% and 91.5% for PEO1 and PEO1TaxR, respectively, as shown in Figs. 2 and 3. We chose a 48 h time-point as at 72 h of treatment the levels of apoptosis in the control untreated cells were significant due to plateau phase growth and confluence of cells in culture. Tables 2 and 3 show similar data obtained for the PEO1 and PEO1CarboR cells in experiments involving treatment with cisplatin, GW2974A singly and in combination.

Table 2 – Data obtained using the Annexin V assay showing different phases of cell death (%) for PEO1 cells treated with GW2974A and cisplatin in combination for 48 h

Treatment	Late	Mid	Early	Viable
Control	2.2 (0.9)	2.0 (0.1)	2.2 (1.3)	93.8 (1.3)
GW2974	4.5 (2.7)	6.0 (4.0)	11.5 (2.0)	76.0 (6.1)
Cisplatin	6.0 (5.2)	3.0 (2.3)	21.5 (9.6)	68.8 (13.8)
Cisplatin + GW2974	7.0 (2.5)	9.7 (4.5)	12.3 (9.2)	71.0 (6.9)

Figures shown are the mean percentages and in parentheses are the S.D. for >3 repeat experiments.

Table 3 – Data obtained using the Annexin V assay showing different phases of cell death (%) for PEO1CarboR (carboplatin resistant) cells treated with GW2974A and cisplatin alone and in combination for 48 h

Treatment	Late	Mid	Early	Viable
Control	3.3 (1.6)	2.5 (1.5)	2.8 (2.5)	91.5 (3.9)
GW2974	11.7 (1.2)	7.3 (6.8)	3.3 (1.1)	77.7 (8.9)
Cisplatin	6.7 (3.1)	7.0 (2.1)	5.7 (4.0)	80.3 (9.3)
GW2974 + cisplatin	15.0 (4.2)	7.3 (6.1)	5.3 (4.9)	72.7 (7.7)

Footnote as for Table 2.

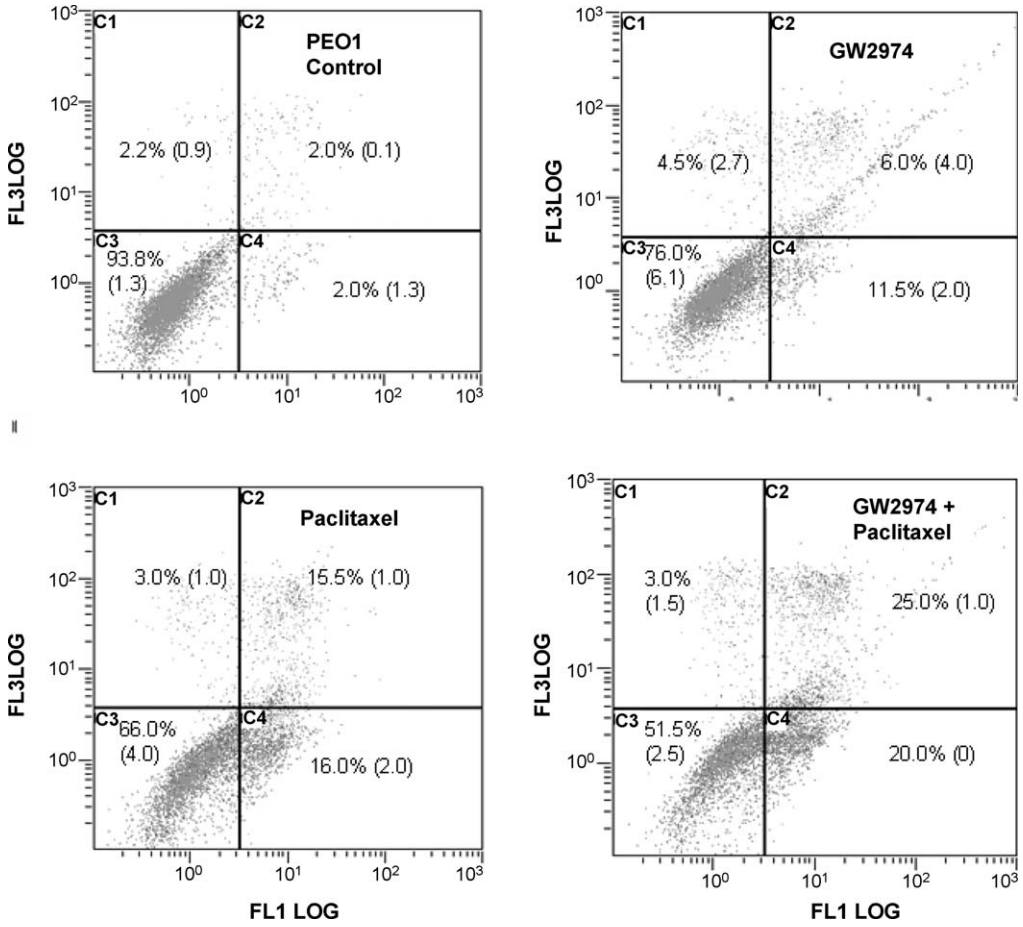

Fig. 2 – Representative flow cytometry data showing apoptosis in drug treated PEO1 parental cells, using the Annexin V assay. Drug doses used are indicated in Methods (above). Top left quadrant is indicative of PI positive cells indicative of late stage apoptosis/necrosis. Top right indicates intermediate stage apoptosis; bottom left indicates live Annexin/PI negative cells and bottom right shows early stage Annexin V positive cells. The data values obtained for >3 repeat experiments have been shown in the panels as % of the total population, in the same manner as for Tables 2 and 3.

3.4. *Levels of ERK-P and Survivin in drug treated PEO1 cell lines*

Fig. 4 shows PEO1 and PEO1CarboR cell lines treated with cisplatin, or paclitaxel in the case of PEO1TaxR, and GW2974A singly and in combination. For PEO1 parental cells cisplatin treatment showed a reduction in levels of ERK2 phosphorylation (44/42 kDa): densitometric scanning showed p44 to be reduced to 0.45 relative to control value of 1.00. For treatment with GW2974A and the combination values for P44 and P42 were very similar to control values (0.98, 1.03 and 0.75 and 1.13, respectively). Effects for the combination with paclitaxel in PEO1 cells were very similar to those seen for cisplatin (data not shown). Interestingly, levels of P-ERK in PEO1CarboR appeared to rise with either GW2974A or cisplatin treatment, over the 48 h time period examined. For the P44 band cisplatin treatment caused an increase to 1.24, GW2974A 1.77 and for P42, the levels were 1.22 and 1.43, respectively, with bands for PEO1CarboR control cells corrected to a value of 1.00. However, significantly, levels of P-ERK were reduced for the combination treatment of GW with cisplatin, p44 0.67 and p42 0.86. The most significant reduction in levels of ERK1/2 phosphorylation were seen for the combination of GW2974A with paclitaxel in the PEO1TaxR cell line. P44/P42 band values for densitometric scanning varying little for the paclitaxel and GW2974A treatments alone (0.91, 0.82 and 0.95, 0.97 for P44 and P42 bands, respectively, compared with PEO1TaxR cells). Significantly, the values for the P44/P42 bands with the combination treatment were 0.0 and 0.39, respectively.

Survivin expression in drug treated PEO1 and PEO1CarboR cells, as shown in Fig. 5, showed a reduction when cells were treated with either GW2974A, cisplatin or the combination of GW2974A with cisplatin. Considering levels expressed in control untreated cells (densitometric scan value for PEO1-CarboR 1.67), relative to the parental PEO1 cells (densitometric scan value 1.00), the expression of Survivin was more reduced in PEO1CarboR cells following GW2974A treatment alone: 0.47 densitometric scan *versus* 1.00 for control untreated, and the combination treatment with cisplatin: 0.46. The Survivin level for PEO1CarboR cells treated with cisplatin was 0.83 *versus* 1.00 for control cells. A similar effect was demonstrated for PEO1TaxR cells (data not shown) under similar treatment conditions. For the PEO1 parental cells densitometric scan results were cisplatin 0.77, GW2974A 0.65 and combination

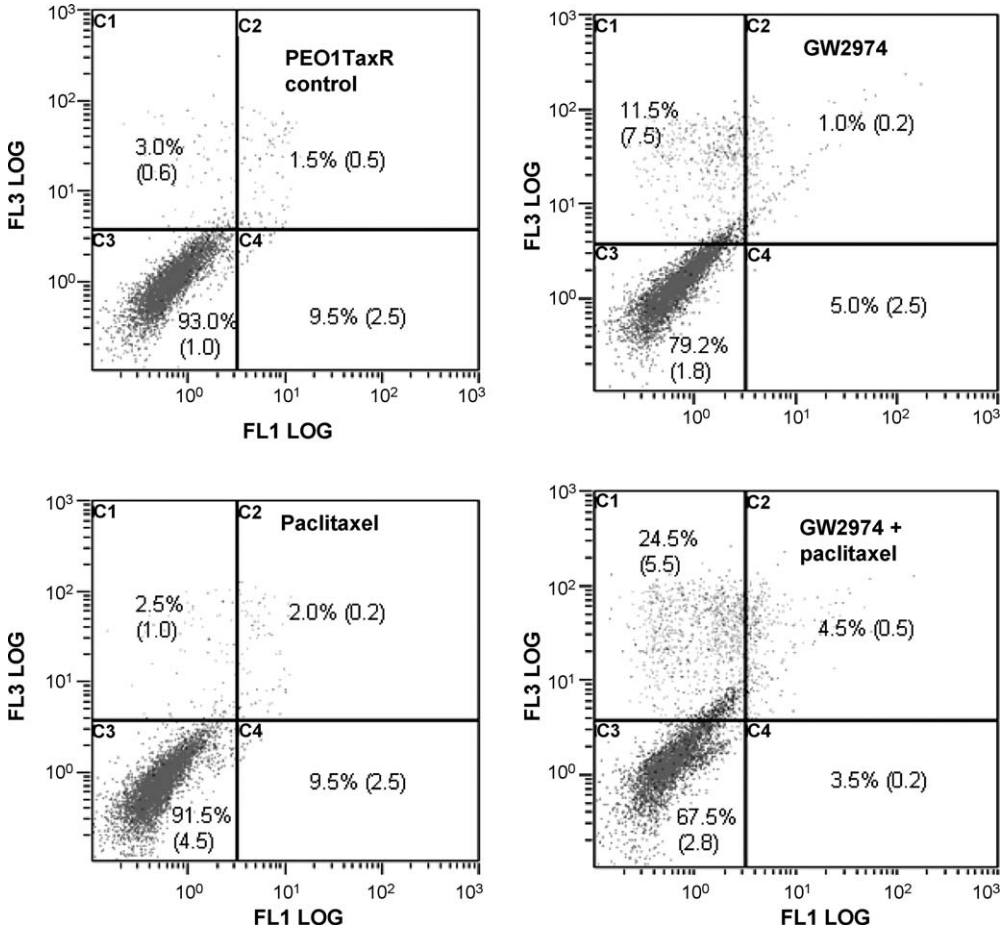

Fig. 3 – Representative flow cytometry data showing apoptosis in drug treated PEO1TaxR cells. Other notes as for legend to Fig. 2.

0.62. Hence, the Survivin levels did not exactly match the levels of apoptosis indicated by Annexin V assay results. However, overall the Survivin levels measured with the drug combination treatments indicated an enhanced effect for the drug resistant PEO1 cells.

4. Discussion

The importance of EGFR in ovarian cancer is highlighted by the fact that up to 70% of cases may show overexpression and it has been implicated in the progression of this disease. Evidence of autocrine and paracrine regulation via TGFα/EGFR activation in ovarian cancer cell lines has also been demonstrated [14]. The present study and work of others [15] suggest that acquired resistance to cytotoxic agents may be associated in part with an upregulation in EGFR expression. Whilst we saw no evidence for increased sensitivity to the EGFR inhibitor GW2974A in the presence of EGFR upregulation, we provide clear evidence for a synergistic effect when the small molecule inhibitor and cytotoxic agent are used in combination, in agreement with the findings of others [15,16]. We saw a clear effect on apoptosis and decreasing levels of the IAP Survivin with the combination schedule. Survivin was chosen as an end-point to study as it is an IAP that is cancer cell specific and we also provide evidence for higher levels of this protein in the

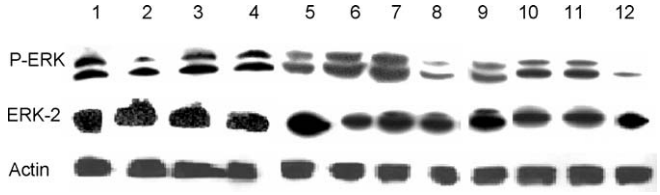

Fig. 4 – Western immunoblotting of PEO1 cell lines for expression of ERK and phosphorylated ERK. Lane 1: PEO1 control; lane 2: cisplatin 0.5 μM; lane 3: GW2974A 20 μM; lane 4: combination cisplatin/GW2974A treatment; lane 5: PEO1CarboR control; lane 6: cisplatin 4 μM; lane 7: GW2974 20 μM; lane 8: combination cisplatin/GW2974A; lane 9: PEO1TaxR control; lane 10: paclitaxel 5 nM; lane 11: GW2974A 20 μM; lane 12: combination paclitaxel/GW2974A.

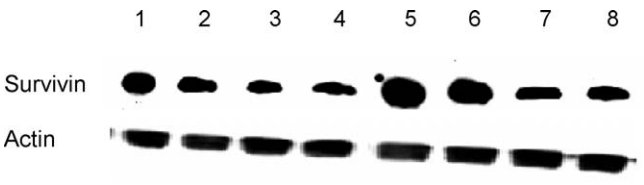

Fig. 5 – Western immunoblotting of PEO1 cell lines for expression levels of Survivin. Lane 1: PEO1 control; lane 2: cisplatin 0.5 μM; lane 3: GW2974A 20 μM; lane 4: combination cisplatin/GW2974A; lane 5: PEO1CarboR control; lane 6: cisplatin 4 μM; lane 7: GW2974 20 μM; lane 8: combination cisplatin/GW2974A.

drug resistant variant cell lines. The observation that EGFR inhibition may not result in a significant anti-proliferative response in EGFR overexpressing ovarian cancer cells has been reported by others [17].

Sewell et al. [9] have demonstrated that gefitinib may reverse EGFR tyrosine phosphorylation and also levels of ERK1/2 induced by TGFα activation in PEO1 cells. Whilst we did not use the approach of activating with either TGFα or EGF, we saw appreciable levels of the ERK p44/42 phospho-protein constitutively expressed in both parental and drug resistant cells. The extent of reversal of p-ERK seen with treatment with GW2974A was modest, but the biggest reductions were seen when the small molecule RTK inhibitor was combined with cisplatin and, particularly, with paclitaxel. GW2974A, like GW2016 was developed as a dual erbB-2/EGFR inhibitor. The IC_{50} values for inhibition of EGFR and erbB-2 by GW2016 measured by inhibition of phosphorylation of a peptide substrate [8], with GW2974A showing somewhat less potency in similar experiments (unpublished data). Interestingly, recent data obtained in our laboratory using an in situ hybridisation technique have indicated that the PEO1 panel of cell lines used in the present study show no variation in their *erbB-2* status. Hence, we may be able to ascribe the activity of this small molecule inhibitor to its effects on EGFR rather than erbB-2 in our cell line panel.

Recently, gefitinib was used in a phase III randomised trial in combination with platinum-based chemotherapy in non-small cell lung cancer. However, the limited efficacy seen may have been due to recruitment of a non-ideal population of patients and highlights the difficulties encountered when dealing with a single-target based therapy such as gefitinib. In order to best optimise the benefit of agents like gefitinib or lapitinab, using relevant combinations of different mechanism based therapies may be a possible option [18]. Another issue to consider with agents such as Glivec® or gefitinib is that resistance to these agents is becoming apparent, which is an inevitable consequence of the unstable genome associated with the cancer cell.

A recent study by Schilder et al. [19] describes a phase II trial using gefitinib in 27 patients with relapsed or persistent ovarian cancer. Interestingly, an increase in progression free survival was correlated with increased EGFR. There was no explanation for the association of this prognostic indicator with longer progression free survival and those particular patients had evidence of platinum-resistance. Eleven *versus* four patients showed progressive disease following gefitinib treatment. The overall response was minimal with one patient showing a partial response possessing a mutation on the tyrosine kinase domain to *EGFR*. The earlier report by Lynch et al. [20] highlighted the importance of specific mutations of the *EGFR* gene in the clinical responsiveness of a small subset non-small cell lung cancers to gefitinib. The mutations were shown to be around the ATP-binding pocket of the tyrosine kinase domain and give rise to enhanced tyrosine kinase activity when ligand activated by EGF, but also to exquisite sensitivity to gefitinib. It remains unknown if the drug resistant PEO1 cell lines used in the present study contain activating mutations in EGFR kinase.

In conclusion, we provide data to support the idea for the combined use of RTK inhibitors with chemotherapeutic agents. It is unclear whether pre-treated ovarian cancers deemed chemo-resistant show enhanced sensitivity to RTK inhibitors when compared with chemotherapy-naive tumours. However, evidence from the present study and from those of others [15] suggest the approach of combining RTK inhibitors with cytotoxic agents should be considered in the management of ovarian cancer.

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