Pergamon

European Journal of Cancer, Vol. 34, No. 7, pp. 1086–1090, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(98)00043-4

Original Paper

Inhibition of Growth of Primary Human Tumour Cell Cultures by a 4-Anilinoquinazoline Inhibitor of the Epidermal Growth Factor Receptor Family of Tyrosine Kinases

B.C. Baguley, E.S. Marshall, K.M. Holdaway, G.W. Rewcastle and W.A. Denny

Cancer Research Laboratory, University of Auckland School of Medicine, Private Bag 92019, Auckland, New Zealand

The epidermal growth factor receptor (EGFR) is thought to mediate the action of the mitogens EGF and tumour growth factor-α (TGF-α) in a variety of cancers, including those of the lung, breast and ovary. A number of new selective inhibitors of EGFR tyrosine kinase have now been developed as potential new antitumour agents. We used a potent inhibitor of this tyrosine kinase, 6-amino-4-[(3-bromophenyl)amino]-7-(methylamino)quinazoline (SN 25531; PD 156273), to determine the responses of primary cultures derived from patients with cancer of the lung, ovary, breast, cervix and endometrium. Cells were cultured in 96-well plates and proliferation assessed by incorporation of ^{3}H-thymidine. Measured growth inhibitory concentrations IC_{50} values) varied from 1 nM to 14 μM with a 1000-fold differential between sensitive and resistant cultures. Results were compared with rates of proliferation, estimated using a paclitaxel-based method. We also measured the IC_{50} values for the tyrosine kinase inhibitor using a number of established human cell lines, and compared them with EGFR content using fluorescent antibody staining and flow cytometry. The presence of EGFR was found to be necessary, but not sufficient, for *in vitro* response. Only a small number of cell lines (3 of 7 for lung, 1 of 7 for ovarian, 2 of 3 squamous cell and 0 of 12 for melanoma) were sensitive to the tyrosine kinase inhibitor. In contrast, 40 of the 50 primary cultures (including 14 of 15 lung cancer samples and 14 of 19 ovarian cancer samples) were sensitive. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: lung cancer, ovarian cancer, doubling time, paclitaxel, flow cytometry, thymidine
Eur J Cancer, Vol. 34, No. 7, pp. 1086–1090, 1998

INTRODUCTION

THE EPIDERMAL growth factor receptor (EGFR) family is thought to mediate the mitogenic action of epidermal growth factor (EGF) in epithelial tissues. Binding of growth factor to EGFR activates its cytoplasmic tyrosine kinase, setting in motion a cascade of signals that are necessary for stimulation of cellular proliferation and suppression of apoptosis [1, 2]. Expression of EGFR and secretion of tumour growth factor-α (TGF-α), which activates EGFR, provides an autocrine growth signal for a variety of cancers, including those of the lung, breast and ovary [3, 4]. The EGFR family, thus provides new targets for anticancer therapy. Quercetin, thiazolidinediones, tyrphostins and quinazoline derivatives have been shown to inhibit EGFR tyrosine kinase activity [5–10]. Some of these inhibitors are exquisitely selective in an isolated enzyme system, possessing 50% inhibitory concentrations of less than 10 pM [10].

At least one inhibitor of EGFR tyrosine kinase is being tested in clinical trials [11], raising the question of whether patients' tumours could be tested individually for response to this therapy. This approach is well established with anti-oestrogens, such as tamoxifen, where the status of oestrogen receptors on tumour cells can be used to individualise treatment [12]. At least some cell lines have been shown to respond *in vitro* to inhibitors of EGFR tyrosine kinase [13–17].

In this paper, we explore the possibility that culturing tumour cells from cancer patients in the presence of an inhibitor of EGFR provides a method of selecting patients who might respond to treatment. We have previously developed

Correspondence to B.C. Baguley.
Received 30 Jul. 1997; revised 4 Dec. 1997; accepted 15 Dec. 1997.

methods for the short-term culture of cells from surgical specimens from melanoma [18] and ovarian cancer [19], with the aim of determining responses to cytotoxic agents. We have shown that the response to the anticancer drug paclitaxel provides information not only on drug sensitivity but also on culture doubling time [19]. In this study, we used 6-amino-4-[(3-bromophenyl)amino]-7-(methylamino)quinazoline (SN 25531; PD 156273; structure shown in Figure 1), a potent inhibitor of EGFR tyrosine kinase [10], to determine whether primary cultures of human cancer tissue can be classified as EGF-dependent or EGF-independent according to their response *in vitro*. We utilised surgical samples from a number of carcinomas, including those of the breast, ovary, cervix and lung. We also carried out studies using human tumour cell lines, in conjunction with flow cytometric analysis of surface EGFR, to determine whether the presence of such receptors is related to the effects of the inhibitor. These results allow comparison between the responses of primary tumours and established cell lines.

MATERIALS AND METHODS

Tumour samples

Formal consent was obtained from all patients, using guidelines approved by the Auckland Area Health Board Ethics Committee. Tissue was obtained by the pathologist from tumours removed at surgery and was immediately sent to the histopathology laboratory where a portion was placed into growth medium. This contained α-MEM supplemented with insulin (10 μg/ml), transferrin (10 ng/ml), sodium selenite (10 ng/ml), penicillin (100 Units/ml), streptomycin (100 μg/ml) and fetal bovine serum (FBS; 5%). Solid tumour specimens obtained from the pathologist were disaggregated immediately or after overnight storage at 4°C. In one experiment, a suspension of fibroblasts was also prepared from normal tissue surrounding the tumour. Normal, adipose, or grossly necrotic material was removed and the tumour tissue was minced finely at room temperature using crossed scalpels. The mince was pushed through a stainless steel screen (0.65 mm mesh size) using a round-ended dental probe. The tumour cells, which were present as small cellular aggregates, were partially enriched by repeated gentle centrifugation (twice at 100 *g* for 5 min and once 25 *g* for 2 min) and resuspension in growth medium. The process was carefully monitored by phase contrast microscopy. Cytospins were prepared and examined by a histopathologist to ensure that the aggregates contained mainly tumour cells.

Chemosensitivity assay

Cells in the tumour aggregates were counted in a haemocytometer to provide an estimate of total cell density. Plates (96-well) were coated with agarose, as previously described [18], in order to prevent growth of fibroblasts. Cultures were set up to contain approximately 940, 1880, 3750 or 7500 cells/well in a volume of 0.15 ml. The different cell densities were required to ensure that there was a linear relationship between cell dose and proliferation. Cultures were incubated with growth medium containing various concentrations of either EGFR kinase inhibitor or paclitaxel, for 7 days. Proliferation was assessed by addition of [^{3}H-methyl]-thymidine (0.04 μCi per well, 0.1 μM final concentration), together with 5-fluorodeoxyuridine (0.1 μM, to prevent endogenous synthesis), 24 h prior to harvest [19]. IC_{50} values (defined as the drug concentrations causing 50% inhibition of thymidine incorporation as compared with that of control cultures) were determined as previously described [20]. In some cases, separate cultures were set up to estimate proliferation. They were incubated for 7 days and labelled with ^{3}H-thymidine for the final 24 h. Cytospins, were then prepared for radioautography by the emulsion dipping method and the proportion of labelled cells measured. The labelled cells were also identified cytologically by a histopathologist.

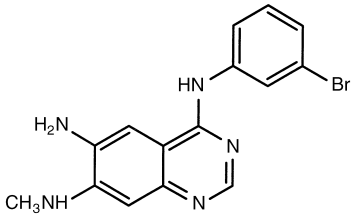

Figure 1. Structure of the tyrosine kinase inhibitor 6-amino-4-[(3-bromophenyl)amino]-7-(methylamino)quinazoline.

Cell lines

The melanoma cell lines SKMEL-5, MALME-3M, LOX-IMVI, the ovarian lines OVCAR-4, OVCAR-5, OVCAR-8 and SKOV-3, the lung line H460 and the cervix line A431 were kindly provided by Mr Richard Camalier, National Cancer Institute, U.S.A. New Zealand melanoma cell lines were developed from pathologically confirmed malignant melanoma. The origins of the cell lines, some of which have been reported previously [19, 20] are described in Table 1. Cell lines were cultured with the inhibitor in 96-well plates for 5 days and proliferation was assessed by incorporation of ^{3}H-thymidine added over the final 6 h.

EGFR expression

Cell lines were tested for high affinity EGFR content by labelling with anti-EGFR antibody by a modification of a previously published method [21]. Cells were lifted from the culture plate using growth medium containing 2 mM ethylene diamine tetra-acetic acid (EDTA) and disaggregated by passage through a 26G needle. The cells were diluted with phosphate buffered saline (PBS) containing 2% FBS (PBS/FBS), centrifuged and washed in PBS/FBS. Samples of 10^6 cells were suspended in a solution containing anti-EGFR antibody (DAKO M886; DAKO Corporation, California, U.S.A. diluted 1:10 in PBS/FBS and left for 45 min on ice, according to the manufacturer's recommendations. Control cell samples were suspended with the isotype control antibody (DAKO X927) under similar conditions. Samples were then washed and incubated with a secondary antibody (FITC IgG, Tago 6240; Tago Inc., California, U.S.A.) for a further 45 min, centrifuged, resuspended in 200 μl PBS/FBS and analysed on a Beckton Dickinson FACScan. Samples were gated on forward and side scatter in order to analyse single cells for EGFR content. Cell frequency was plotted against fluorescence intensity and the ratio of median fluorescence intensities of the anti-EGFR antibody and control antibody treated suspensions was calculated.

RESULTS

Response of primary cultures to EGFR tyrosine kinase inhibitor

A high success rate (> 80%) was obtained in the culture of tumour cells from patients with a variety of cancer types. Cultures were obtained from cancer of the ovary (19), lung

(15), endometrium (four), cervix (four) and breast (three), pancreas (one), colon (one), omentum (one) and fallopian tube (one). There was also one glioma. Twenty-nine of the cultures were tested at the end of the assay by autoradiography and cytology. The labelled cells were identified as tumour cells and the proportion of labelled cells was found to vary between < 1 and 70% (median 10%). All cultures were assayed for growth inhibition by the tyrosine kinase inhibitor and by paclitaxel. A high proportion (40 of 50) of the primary cultures (including 14 of 15 lung cancer samples and 14 of 19 ovarian cancer samples) were sensitive to the inhibitor (Figure 2). A primary culture of fibroblasts was also tested and found to be insensitive. Culture doubling times, estimated from the paclitaxel data using a previously developed method [19], were found to vary from 57 h to approximately 7 weeks (Figure 3). Clinical details of patients in this study will be described in a separate publication.

Response of cell lines to EGFR tyrosine kinase inhibitors

The response to the tyrosine kinase inhibitor of 29 cell lines, 19 of which were early passage cell lines (< 25 passages) was determined. None of the 12 melanoma cell lines of the panel were sensitive to the inhibitor, with one (LOX) found to be reproducibly stimulated by the presence of the inhibitor (up to 6-fold, depending on the drug concentration). Of the cell lines, only six of 29 were sensitive to the tyrosine kinase inhibitor (Figure 2), significantly less than for primary cultures ($P < 0.001$). Three of seven of the lung lines, one of seven of the ovarian lines and two of three of the squamous cell lines responded (Figure 2). The 1,000-fold range of IC_{50} values (20 nM to 14 μM) enabled cell lines to be classified as either sensitive or resistant to the inhibitor. IC_{50} values for a series of nine other analogues of the inhibitor showed a similar pattern (results not shown). The estimated culture doubling times for the lines was in general much shorter than those of the primary cultures (Figure 3).

The presence of EGFR for a panel of cell lines was determined by flow cytometry and was detectable in all cell lines, except the melanoma lines. A number of lines exhibiting moderate to high numbers of receptors were found to be insensitive to the inhibitor (Figure 4). The primary fibroblast culture was also tested and found to contain detectable EGFR.

Table 1. *Tumour sources of cell lines*

Line	Age (years)	Sex	Tumour
NZSQ1	45	M	Poorly differentiated squamous cell carcinoma metastatic to cervical lymph nodes
NZV1	83	F	Poorly differentiated squamous cell carcinoma of vulva
NZL1	63	M	Adenocarcinoma of lung
NZL2	79	F	Poorly differentiated adenocarcinoma of lung
NZL3	55	F	Poorly differentiated grade III adenocarcinoma of lung
NZL5	62	M	Well differentiated mesothelioma of lung
NZL6	70	M	Poorly differentiated adenocarcinoma of lung
NZOV1	57	F	Moderately differentiated grade III papillary serous cystadenocarcinoma of ovary
NZOV2	81	F	Poorly differentiated grade III serous adenocarcinoma of ovary
NZOV4	59	F	Adenocarcinoma of ovary
NZM1	47	M	Malignant melanoma metastatic to obturator lymph node
NZM2	47	M	Malignant melanoma metastatic to inguinal lymph node
NZM3	69	M	Malignant melanoma metastatic to subcutis of neck
NZM4	56	M	Metastatic malignant melanoma, (peritoneal effusion)
NZM5	79	M	Malignant melanoma metastatic to axillary lymph node
NZM7	36	M	Malignant melanoma metastatic to cervical lymph node
NZM9	80	M	Malignant melanoma metastatic to axillary lymph node
NZM6	72	F	Malignant melanoma metastatic to small bowel
NZM10	52	M	Malignant melanoma metastatic to lung

DISCUSSION

The results demonstrate a clear demarcation among primary cultures of tumour cells into those that are either sensitive or resistant to a phenylquinazoline-based inhibitor of EGFR tyrosine kinase. A 1,000-fold differential in IC_{50} values is apparent for most of the primary cultures, consistent with the hypothesis that the inhibitor is arresting cell growth as a consequence of inhibiting EGFR-dependent signal transduction. The high success rate of culture reflects an altered technical approach, where the tumour cells are grown as

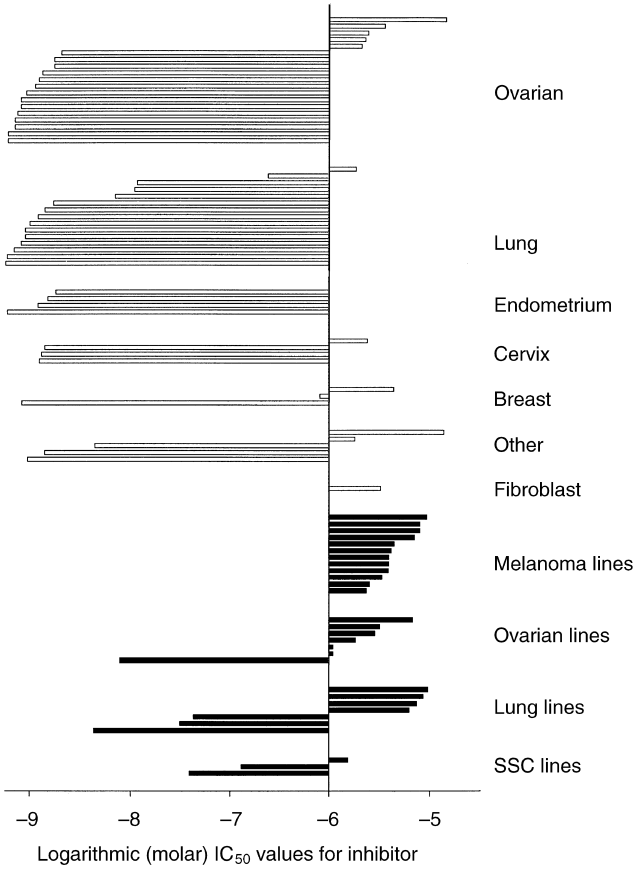

Figure 2. IC_{50} values for the tyrosine kinase inhibitor using primary cultures of tumour cells from patients with carcinomas (open bars) and established cell lines (solid bars). The types of tumours are shown at the right of the graph.

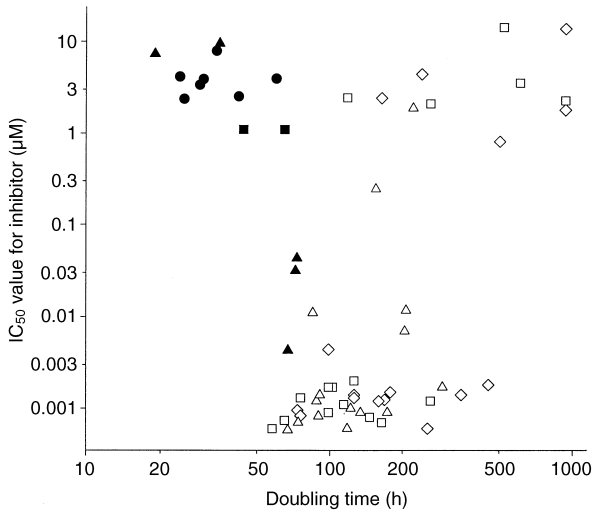

Figure 3. Relationship between estimated culture doubling times and IC_{50} values for the tyrosine kinase inhibitor. Primary cultures include ovarian carcinoma (□), lung carcinoma (△) and other tumours (◇). Established tumour cell lines include melanoma (●), ovarian carcinoma (■), lung carcinoma (▲) and squamous cell carcinoma (◆).

small aggregates (generally 10–50 cells per aggregate and small enough to be pipetted during set-up of cultures), rather than as a single cell suspension. In our experience, the success rate of primary cultures of carcinomas, as well as the degree of thymidine incorporation, is much higher when the tumour cells are cultured in such a way, allowing at least some of the tumour architecture and extracellular matrix components to remain. The extracellular matrix and associated integrins may be important for short-term substrate-independent growth [22].

It is also possible to categorise established cell lines into those that are sensitive or resistant to the tyrosine kinase inhibitor. The IC_{50} values for the sensitive lines are approximately 10-fold higher than those for the sensitive primary cultures (Figure 2). The reason for the lower differential, in comparison with the primary cultures, is not clear, but may result from the fact that the primary cultures grow on a layer of agarose [18], while the established cell lines grow directly on the culture plate surface. These cell lines were also cultured on agarose-coated plates, but were found not to grow (results not shown), suggesting that they have adapted to growth on plastic surfaces.

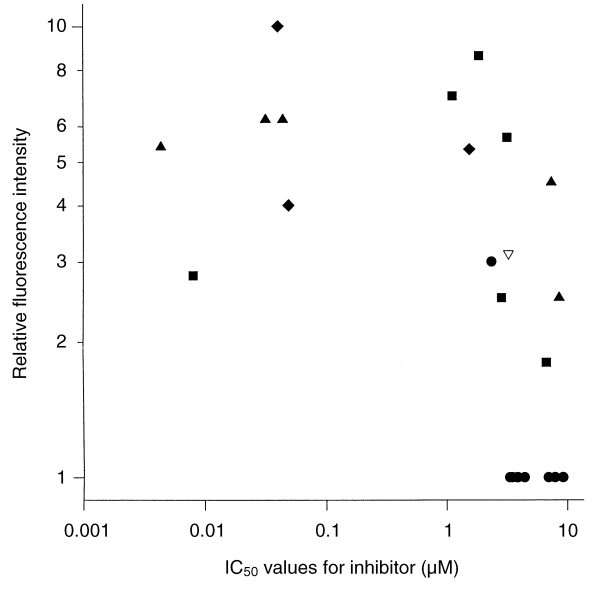

Figure 4. Relationship between epidermal growth factor receptor (EGFR) content, as measured using fluorescently labelled antibody and flow cytometry and IC_{50} values for the tyrosine kinase inhibitor. Tumour cell lines were derived from melanoma (●), ovarian carcinoma (■), lung carcinoma (▲) and squamous cell carcinoma (◆). A value for normal fibroblasts is also shown (▽).

The response of cell cultures to paclitaxel allows estimation of cell culture doubling times [19] and thus comparison of drug sensitivity and growth rate (Figure 3). In general, primary cultures that are resistant to the tyrosine kinase inhibitor have longer doubling times, although there is considerable overlap. The established cell lines, as expected, have shorter doubling times. A number of the primary cultures were also subject to autoradiography following 24 h exposure to ^{3}H-thymidine on the final day of the culture period. The percentage of labelled cells, which varied from < 1 to 70%, indicates a wide range in the proportion of cycling cells. The percentages are not related to the measured doubling times and probably reflect wide variations in the proportion of differentiated or non-proliferating tumour cells in the samples. The results are consistent with wide variations in S-phase fractions reported for clinical tumour samples, such as those from ovarian cancer [23].

A number of cell lines were labelled for EGFR using a specific antibody and the labelling quantitated by flow cytometry (Figure 4). The presence of EGFR appears to be necessary, but not sufficient, for response to the tyrosine kinase inhibitor. The anti-EGFR antibody labelled none of the melanoma lines, with the exception of LOX, whereas all of the carcinoma lines were labelled. The A431 line, which is known to overexpress EGFR [14], showed the highest intensity of labelling (Figure 4).

EGFR can lead to signal transduction stimulation through at least two pathways. The first is through a complex series of proteins, including Grb-2, Sos, Ras, Raf and the MAP kinase cascade [1]. This pathway initiates gene activation leading to mitogenesis, while a second pathway involves activation of the STAT1 pathway and inhibition of mitogenesis [24]. The balance of these two pathways (and perhaps others) leads to the response of an individual cell line. In this regard, one of the lines tested (LOX) was interesting in that growth, as measured by thymidine incorporation, was reproducibly stimulated by the presence of the inhibitor.

In conclusion, we have shown that a high proportion of primary cultures from patients with carcinomas of the lung, ovary, cervix and breast respond to a specific inhibitor of EGFR tyrosine kinase by cessation of growth. A similar phenomenon is observed in established cell lines, although the proportion of carcinomas responding (6 of 29) is significantly less than that for primary cultures (40 of 50). Labelling of EGFR in cell lines demonstrates that, although all of the carcinomas contained EGFR, not all responded to the EGFR tyrosine kinase inhibitor. If this result applies to the primary cultures, it may mean that immunochemical detection of EGFR in carcinomas will not indicate whether such carcinomas are potentially responsive to tyrosine kinase inhibitors. Culture methods, such as those used here, may be useful for determining responses of individual patients to EGFR tyrosine kinase inhibitors that are likely to be tested clinically in the near future.

1. Bridges AL. The epidermal growth factor receptor family of tyrosine kinases and cancer: can an atypical exemplar be a sound therapeutic target? *Curr Med Chem* 1996, **3**, 211–226.
2. Mendelsohn J, Fan Z. Epidermal growth factor receptor family and chemosensitization. *J Natl Cancer Inst* 1997, **89**, 341–343.
3. Harris AL. The epidermal growth factor receptor as a target for therapy. *Cancer Cells* 1990, **2**, 321–323.
4. Rabiasz GJ, Langdon SP, Bartlett JMS, *et al.* Growth control by epidermal growth factor and transforming growth factor-alpha in human lung squamous carcinoma cells. *Br J Cancer* 1992, **66**, 254–259.
5. Fry DW, Kraker AJ, McMichael A, *et al.* A specific inhibitor of the epidermal growth factor receptor tyrosine kinase. *Science* 1994, **265**, 1093–1095.
6. Ward WHJ, Cook PN, Slater AM, Davies DH, Holdgate GA, Green LR. Epidermal growth factor receptor tyrosine kinase. Investigation of catalytic mechanism, structure-based searching and discovery of a potent inhibitor. *Biochem Pharmacol* 1994, **48**, 659–666.
7. Levitzki A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science* 1995, **267**, 1782–1788.
8. Rewcastle GW, Denny WA, Bridges AJ, *et al.* Tyrosine kinase inhibitors. 5. Synthesis and structure–activity relationships for 4-[(phenylmethyl)amino]- and 4-(phenylamino)quinazolines as potent adenosine 5′-triphosphate binding site inhibitors of the tyrosine kinase domain of the epidermal growth factor receptor. *J Med Chem* 1995, **38**, 3482–3487.
9. Denny WA, Rewcastle GW, Bridges AJ, Fry DW, Kraker AJ. Structure–activity relationships for 4-anilinoquinazolines as potent inhibitors at the ATP site of the epidermal growth factor receptor *in vitro*. *Clin Exp Pharm Physiol* 1996, **23**, 424–427.
10. Bridges AJ, Zhou H, Cody DR, *et al.* Tyrosine kinase inhibitors. 8. An unusually steep structure–activity relationship for analogues of 4-(3-bromoanilino)-6,7-dimethoxyquinazoline (PD 153035), a potent inhibitor of the epidermal growth factor receptor. *J Med Chem* 1996, **39**, 267–276.
11. Woodburn JR, Barker AJ, Gibson KH, *et al.* ZD 1839, an epidermal growth factor tyrosine kinase inhibitor selected for clinical development. *Proc Am Assoc Cancer Res* 1997, **38**, 623.
12. Sutherland RL, Lee CSL, Feldman RS, Musgrove EA. Regulation of breast cancer cell cycle progression by growth factors, steroids and steroid antagonists. *J Steroid Biochem Mol Biol* 1992, **41**, 315–321.
13. Reddy KB, Mangold GL, Tandon AK, *et al.* Inhibition of breast cancer cell growth *in vitro* by a tyrosine kinase inhibitor. *Cancer Res* 1992, **52**, 3636–3641.
14. Brunton VG, Carlin S, Workman P. Alterations in EGF-dependent proliferative and phosphorylation events in squamous cell carcinoma cell lines by a tyrosine kinase inhibitor. *Anti-Cancer Drug Design* 1994, **9**, 311–329.
15. Buchdunger E, Trinks U, Mett H, *et al.* 4,5-Dianilinophthalimide—a protein-tyrosine kinase inhibitor with selectivity for the epidermal growth factor receptor signal transduction pathway and potent *in vivo* antitumor activity. *Proc Natl Acad Sci USA* 1994, **91**, 2334–2338.
16. Han YC, Caday CG, Nanda A, Cavenee WK, Huang HJS. Tyrphostin AG 1478 preferentially inhibits human glioma cells expressing truncated rather than wild-type epidermal growth factor receptors. *Cancer Res* 1996, **56**, 3859–3861.
17. Jones HE, Dutkowski CM, Barrow D, Harper ME, Wakeling AE, Nicholson RJ. New EGF-R selective tyrosine kinase inhibitor reveals variable growth responses in prostate carcinoma cell lines PC-3 and DU-145. *Int J Cancer* 1997, **71**, 1010–1018.
18. Marshall ES, Finlay GJ, Matthews JHL, Shaw JHF, Nixon J, Baguley BC. Microculture-based chemosensitivity testing: a feasibility study comparing freshly explanted human melanoma cells with human melanoma cell lines. *J Natl Cancer Inst* 1992, **84**, 340–345.
19. Baguley BC, Marshall ES, Whittaker JR, *et al.* Resistance mechanisms determining the *in vitro* sensitivity to paclitaxel of tumour cells cultured from patients with ovarian cancer. *Eur J Cancer* 1995, **31A**, 230–237.
20. Marshall ES, Holdaway KM, Shaw JHF, Finlay GJ, Matthews JHL, Baguley BC. Anticancer drug sensitivity profiles of new and established melanoma cell lines. *Oncol Res* 1993, **5**, 301–309.
21. Brockhoff G, Hofstaedter F, Knuechel R. Flow cytometric detection and quantitation of the epidermal growth factor receptor in comparison to Scatchard analysis in human bladder carcinoma cell lines. *Cytometry* 1994, **17**, 75–83.
22. Meredith JE, Fazeli B, Schwartz MA. The extracellular matrix as a cell survival factor. *Mol Biol Cell* 1993, **4**, 953–961.
23. Alama A, Chiara S, Merlo F, *et al.* Tumour kinetics, response to chemotherapy and survival in primary ovarian cancer. *Eur J Cancer* 1994, **30A**, 449–452.
24. Quelle FW, Thierfelder W, Witthuhn BA, Tang B, Cohen S, Ihle JN. Phosphorylation and activation of the DNA binding activity of purified stat 1 by the janus protein-tyrosine kinases and the epidermal growth factor receptor. *J Biol Chem* 1995, **270**, 20775–20780.

Acknowledgements—Supported by the Cancer Society of New Zealand, the Health Research Council of New Zealand and the Parke-Davis Division of Warner-Lambert. The authors gratefully acknowledge Drs James Shaw, Tim Christmas, John Whittaker and other hospital staff for help with providing surgical specimens, Drs Ian Smeeton and Margaret Sage for help with histopathology, and the cancer patients for their participation. We also thank Dr Wilbur R. Leopold and other scientists at Parke-Davis Research, Ann Arbor, Michigan, U.S.A. for their advice and support.