The effect of ZD1839 (IressaTM), an epidermal growth factor receptor tyrosine kinase inhibitor, in combination with cisplatin, on apoptosis in SCC-15 cells

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Abstract. The aim of this study was to determine the effect of ZD1839 on growth and apoptosis in SCC-15 (a human head and neck cancer cell line) alone, or in combination with cisplatin. High expression of the epidermal growth factor receptor has been implicated in the development of squamous cell carcinomas of head and neck. ZD1839 ('Iressa') is an orally active, selective epidermal growth factor receptor tyrosine kinase inhibitor that blocks signal transduction pathways implicated in proliferation and survival of cancer cells, and other host-dependent processes promoting cancer growth. Here, growth arrest was observed with 3.64 μM ZD1839. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay revealed a significant decrease ($P < 0.001$) in the percentage of surviving cells upon treatment with ZD1839 and cisplatin compared with cisplatin or ZD1839 on their own. Combined therapy of 3.64 μM ZD1839 for 24 h, prior to administration of 100 μM cisplatin, significantly ($P < 0.001$) and additively increased the cytotoxicity effect of cisplatin. p53-independent apoptosis was seen with cisplatin treatment, a novel finding. These data support the use of ZD1839 in anti-cancer therapy, and particularly in combination therapy. Cisplatin may induce p53-independent apoptosis. Over-expression of Bcl-2 in head and neck squamous cell carcinoma tumour cell lines is unlikely to be a general mechanism to protect these cells from apoptosis.

INTRODUCTION

The epidermal growth factor receptor tyrosine kinase (EGFR-TK) initiates diverse signal transduction pathways in tumour cells that have a profound effect on their biology. Activation of the EGFR-TK provides signals that drive deregulated proliferation, invasion and metastasis, angiogenesis, and enhanced cell survival (Ritter & Arteaga 2003). Over-expression of human epidermal growth factor receptor (EGFR) has been associated with a variety of human malignancies (Chen *et al.* 2000). EGFR is commonly expressed or over-expressed by many tumours, including those of the head and neck, lung, breast, colon, prostate, ovary and uterine cervix (Klijn *et al.* 1992;

Di Gennaro *et al.* 2003; Caponigro 2004). Over-expression correlates with rapid disease progression, poor response to treatment and poor survival (Baselga 2002; Normanno *et al.* 2003). These data have provided a rationale for targeting this signalling network with novel treatment approaches (Mendelsohn & Baselga 2000).

The treatment applied in this study is with ZD1839 ('Iressa') an orally active selective EGFR-TK inhibitor. Phase III clinical trials are currently evaluating ZD1839 in combination with gemcitabine/cisplatin or paclitaxel/carboplatin as first-line treatment of non-small cell lung cancer and an ongoing programme of clinical trials is investigating the use of further combinations against a variety of tumours (head and neck, prostate, colon and breast) (Herbst 2002; Cohen *et al.* 2003; Daneshmand *et al.* 2003; Schiller 2003; Vicentini *et al.* 2003).

Most head and neck cancers are deficient in p53 function because of mutation or deletion. Studies have shown that functional loss of p53 can induce apoptosis in a variety of cancer cells (Hague *et al.* 1997; Patel *et al.* 2000; Choi *et al.* 2001; Magne *et al.* 2002), thus indicating that apoptosis involves a p53-dependent as well as a p53-independent pathway. The purpose of this study was to examine the efficacy of ZD1839 to cause apoptosis, alone and in combination with cisplatin.

MATERIALS AND METHODS

Cells of the SCC-15 cell line (squamous cell carcinoma of the tongue, American Type Culture Collection, ATCC CRL 1623) were grown in Dulbecco's modified Eagle's medium (DMEM)/nutrient mix F12 (GibcoBRL, Paisley, UK) medium containing 10% fetal calf serum (FCS; GibcoBRL), penicillin–streptomycin 1 ml/100 ml (Sigma Chemical Co., Poole, UK) and hydrocortisone 100 µl/100 ml (Sigma Chemical Co.) in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Cells were treated with ZD1839 (AstraZeneca, Macclesfield, UK) made up to 50 mM in neat dimethylsulfoxide (DMSO) and diluted to 3.64 µM for use. Cells were also separately treated with 10, 50 and 100 µM cisplatin solution (Faulding Pharmaceutical PLC., Royal Leamington Spa, UK).

Cisplatin and ZD1839 cell cytotoxicity assay

Cisplatin and ZD1839 cytotoxicity were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Chemical Co.).

A range of concentrations of cisplatin (5, 10, 30, 60, 100, 150, 200 and 250 µM) was added to SCC-15 cells, left for 2 h, and then rinsed off. Similarly, a range of concentrations of ZD1839 (0.05, 0.08, 0.5, 0.9, 1.5, 2.5, 3.64 and 5 µM) was applied to tumour cell cultures. The total experiment was carried out over 5 days.

Spectrophotometrical absorbance of the samples was measured using a microtitre (ELISA) plate reader at a wavelength of 570 nm. Dilutions were assayed in triplicate and cytotoxicity was calculated using the following equation:

$$\% \text{ Cytotoxicity} = 100(a - b)/a$$

a = mean absorbance of triplicate wells with culture media only (control); b = mean absorbance of triplicate wells with cisplatin or ZD1839-treated cell cultures.

Cytotoxicity of cisplatin combined with ZD1839 was also determined by use of the MTT assay. Cells (5×10^4) were incubated overnight in 100 µl DMEM/nutrient mix F12 medium

containing 10% FCS in 96-well plates. The next day, 100 µl of three concentrations of ZD1839 (0.08, 2.5 and 3.64 µM) were applied to the cells, now in serum-free medium, and were left for at least 24 h before adding 100 µl each of 10 µM, 50 µM and 100 µM of cisplatin. Specimens were left for 2 h and then the combination drugs were washed off. Percentage cytotoxicity was determined by the MTT assay after 24 and 48 h.

Methyl-green/pyronin staining of SCC-15 cells treated with ZD1839 and cisplatin

SCC-15 cells (2×10^4) were grown on coverslips and were left for 4 days to grow in an exponential manner. ZD1839 (3.64 µM) was added to each coverslip and was left for 24 h. Then, 100 µM cisplatin was added and was left for 2 h before the drug combination was washed off. Cells were then fixed with 10% formalin for 10 min, washed in phosphate-buffered saline (PBS) and stained with methyl-green/pyronin (Al-Hazzaa & Bowen 1998) for 1 h. Untreated (control) samples and samples treated with 3.64 µM ZD1839 only, were fixed and stained after 48 h.

Fluorescence of p53 antibody

SCC-15 cells (1×10^6) were fixed in 2% paraformaldehyde for 1 h at room temperature and then were washed in PBS.

Cell permeabilization

Cells were centrifuged for 5 min at 200 g. The fixed cell pellet was re-suspended in 1 ml 0.2% Tween in PBS for 15 min at room temperature and then 1 ml of PBS supplemented with 2% bovine serum albumin (BSA). NaAz (0.1%) was added and the cells were incubated for 10 min at room temperature. The suspension was then centrifuged for 5 min at 200 g, and cells washed once with PBS. Each pellet was then re-suspended in 1 ml PBS.

Cell staining

For staining of internal antigens, cells were incubated with 1 : 40 dilution (10 µl) anti-p53 mAb conjugated with fluorescein isothiocyanate (FITC) (DAKO, Glostrup, Denmark) for 30 min at 4 °C in the dark. After staining, the cells were washed twice with 2% BSA then resuspended in 1 ml PBS. Negative isotype controls were also prepared by incubating the cells with a FITC-conjugated mouse IgG1 (DAKO).

Sample analysis

Samples were analysed on a Becton Dickinson FACScan flow cytometer incorporating an argon laser tuned at 488 nm. Signals for forward and side light scatter and fluorescence at 488 nm were collected for 10 000 particles. Histograms were prepared using Becton Dickinson software (CELLFIT) on an IBM-compatible PC.

Fluorescence of Bcl-2 antibody

Cell staining: for staining of internal antigens: cells were incubated with 1 : 40 dilution (10 µl) of anti-Bcl-2 mAb conjugated with FITC (DAKO) for 30 min at 4 °C in the dark. After staining cells were washed twice with 2% BSA then resuspended in 1 ml PBS. Negative isotype controls were also prepared by incubating the cells with a FITC conjugated secondary mouse Ab (DAKO, Denmark). Cell permeability and sample analysis was as mentioned for p53.

Statistical analysis

Data were analysed by two-way ANOVA analysis (Fry 1993) using MINITAB software, to assess differences between the four treatment group means (control, cisplatin treatment, ZD treatment

and treatment with the combination of ZD1839 with cisplatin). The effect of cisplatin combined with ZD1839 was compared with the effect of each drug alone. In addition, a non-parametric test (Kruskal–Wallis) to assess the difference between the treatment group medians was performed on the 48-h treatment data (Fry 1993).

RESULTS

A time-course relationship of SCC-15 cell line response to cisplatin treatment was determined. The cytotoxicity of cisplatin was maximal with 100 μM for 48 h (Fig. 1). ZD1839 exhibited a less dramatic time course effect with a maximum at 3.64 μM for 72 h (Fig. 2).

Statistical two-way ANOVA analysis revealed that treatment of SCC-15 cells with either 100 μM cisplatin or 3.64 μM ZD1839 alone resulted in a highly significant difference in survival of cells from the untreated category ($P < 0.001$). However, exposure to 3.64 μM ZD1839 for 24 h, prior to administration of 100 μM cisplatin, significantly ($P < 0.001$) and additively increased the cytotoxicity of cisplatin from $48.5\% \pm 4.9$ to $70.3\% \pm 1.9$ after 24 h and from $43.7\% \pm 8.4$ to $74.1\% \pm 4.2$ after 48 h (Fig. 3). Exposure of the cells to 3.64 μM ZD1839, for 24 h prior to the administration of a lower dose of cisplatin (50 μM), significantly ($P < 0.001$) increased the cytotoxicity of cisplatin after 24 h. Forty-eight hours of treatment also produced a highly significant ($P < 0.001$) difference with interaction of the two treatments (Fig. 4). The analysis also revealed that the combination of 3.64 μM ZD1839 with 10 μM cisplatin significantly ($P < 0.002$) increased cytotoxicity after 24 h. However, interaction of the two treatments at this dosage (10 μM) showed a marginal increase ($P = 0.049$) in cytotoxicity after 48 h (Fig. 4).

In addition, the Kruskal–Wallis test for 48-h treatment of the three concentrations of cisplatin (10 μM , 50 μM and 100 μM) revealed highly significant differences ($P < 0.001$) between the medians of the four groups tested. This result supports the analysis of variance above, and indicates a similar interaction effect of the two treatments on the medians as shown for the means.

Figure 1. Percentage of viable SCC-15 cells after treatment with 100 μM cisplatin. The data represent mean $\pm$ SE of triplicate experiments.

Figure 2. Percentage of surviving SCC-15 cells after treatment with 3.64 μ M ZD1839. The data represent mean $\pm$ SE of triplicate experiments.

Figure 3. Cytotoxicity MTT assay of SCC-15 cells treated with 100 μ M cisplatin and 3.64 μ M ZD1839. The bars represent $\pm$ SE.

Cell morphology studies using methyl-green/pyronin staining of SCC-15 cells revealed a healthy confluent layer of untreated control cells (Fig. 5a). SCC-15 cells treated with 100 μ M cisplatin demonstrated a marked increase in apoptosis and a reduction in cell number (Fig. 5b). Treatment with 3.64 μ M ZD1839 revealed pale pyronin staining, indicating a reduction in RNA content. In addition, there was an 80% reduction in cell growth and no appearance of apoptosis (Fig. 5c). Furthermore, the combination of 100 μ M cisplatin and 3.64 μ M ZD1839 resulted in 75% reduction in cell density. Cells appeared more vacuolated, with a reduced level of pyronin staining. In addition, an increase in apoptosis was observed reaching 20% (Fig. 5d).

SCC-15 cells expressed negligible amounts of p53 protein (Fig. 6). The mean fluorescence of the p53 antibody stain was similar between the negative isotype control and the positive p53 antibody in all categories. However, the SCC-15 cells were found to possess detectable amounts of Bcl-2 (Fig. 7). The mean fluorescence of the Bcl-2 antibody stain consistently revealed a difference between the negative isotype control and the positive Bcl-2 antibody.

Figure 4. Cytotoxicity MTT assay of SCC-15 cells treated with 3.64 μM ZD1839 and various concentration of cisplatin. The bars represent $\pm$ SE.

Figure 5. Light microscope images of SCC-15 cells stained with methyl-green/pyronin. Methyl-green stains nuclei and pyronin stains cytoplasm. Magnification $\times 500$. (a) Control, untreated SCC-15 cells; (b) SCC-15 cells treated with 100 μM cisplatin after 24 h. The apoptotic cells show blebbing of cytoplasm; intense pyronin staining indicates high levels of RNA. Nuclei and nucleolar fragments are stained heavily with methyl-green; (c) SCC-15 cells treated with 3.64 μM ZD1839 after 24 h. Pale pyronin staining of the cytoplasm and reduction in cell number; (d) SCC-15 cells treated with 3.64 μM ZD1839 24 h prior to treatment with 100 μM cisplatin.

Figure 6. Flow cytometric analysis of p53 activity in the SCC-15 cell line, represented by fluorescence histograms after staining with primary anti-p53 mAb conjugated with FITC. The vertical axis defines the cell number and the horizontal axis (FL-1) indicates p53 expression.

Figure 7. Flow cytometric analysis of Bcl-2 protein in the SCC-15 cell line, represented by fluorescence histograms after staining with primary anti-Bcl-2 mAb conjugated with FITC. The vertical axis defines the cell number and the horizontal axis (FL-1) indicates Bcl-2 expression.

DISCUSSION

The cytotoxic effect in this study of ZD1839 on SCC-15 cells reflects an anti-proliferation activity of ZD1839, which is supported by morphological studies with methyl-green/pyronin staining. This effect of ZD1839 on the SCC-15 cell line has also been reported by Di Gennaro *et al.* (1999), when they demonstrated that ZD1839 induced growth arrest in these cells by inhibiting basal and EGF-induced EGFR-mediated signalling. In addition, inhibition of proliferation by ZD1839 in various cell lines has been reported (Campiglio *et al.* 2004; Shintani *et al.* 2004; Sumitomo *et al.* 2004).

The effect of combining EGFR inhibitor ZD1839 with cisplatin in this head and neck squamous cell carcinoma (HNSCC) cell line SCC-15, was to increase growth inhibition in an additive manner. There is good reason to believe that this is an effective combination as we have demonstrated that the combined treatment increased growth arrest and induced apoptosis. The cytotoxic effect of cisplatin was enhanced when in combination with ZD1839. Furthermore this enhancement was also observed with the use of lower doses of cisplatin (50 μM and 10 μM).

It has been reported that ZD1839 and cisplatin in combination have led to an increase in growth inhibition and enhanced apoptotic cell death in ovarian, breast and colon cell lines (Ciardiello *et al.* 1999; Schiller 2003) and in head and neck cancer cell preparations (Magne *et al.* 2003). These data lend support to the development of clinical trials involving this combination of drugs.

Morphological studies of methyl-green/pyronin suggested that SCC-15 cells experienced an apparent reduction in RNA content after application of ZD1839 prior to treatment with cisplatin. Despite this, apoptosis proceeded in the predicted manner.

This study has revealed that ZD1839 has an anti-proliferative effect on SCC-15 cells; this is because ZD1839 is a highly specific EGF receptor kinase inhibitor. Inhibition of EGF receptor transphosphorylation by ZD1839 blocks signal transduction at its first step, thus providing an anti-proliferative effect. Moreover, this activity has been found to be enhanced when ZD1839 was co-administered with the chemotherapeutic agent, cisplatin (Al-Hazzaa *et al.* 2000).

Flow cytometric data on SCC-15 cells revealed that this cell line expressed no p53 protein. This finding has previously been reported by Min *et al.* (1994), when they demonstrated that the SCC-15 cell line expresses negligible amounts of p53 transcript and undetectable levels of p53 protein.

Although cisplatin may initiate apoptosis through pathways modulated by p53, this tumour suppressor gene is not always required for apoptosis (Gonzalez *et al.* 2001). Zamble *et al.* (1998) have reported that cisplatin induces apoptosis in cells expressing either wild-type or mutant p53 protein, or even in cells lacking p53. However, it has been shown that cisplatin treatment in some ovarian cell lines decreases or even abolishes p53 protein levels (Gonzalez *et al.* 2001).

The level of appearance of Bcl-2 expression revealed that this protein did not inhibit apoptosis, as shown by flow cytometric analysis. There are, however, physiological situations in which Bcl-2 expression apparently fails to protect cells from apoptosis. In support, expression of Bcl-2 did not show any relation to apoptosis in a study carried out by Ravi *et al.* (2001) on patients with squamous cell carcinoma of the oral cavity. The results of this study suggest that over-expression of Bcl-2 in HNSCC tumour cell lines is unlikely to be a general mechanism to protect these cells from apoptosis.

In conclusion, ZD1839 induces cell growth arrest in SCC-15 cells. Cisplatin-induced apoptosis *in vitro* can be enhanced by pre-exposure of cells to ZD1839, an EGFR-TK inhibitor, despite the loss of p53 function. Therefore, it is important, especially from the therapeutic point of view,

to recognize that there are p53-independent cell death pathways induced by cisplatin which can be enhanced by the combined therapy of cisplatin with ZD1839. The results of this study suggest that cell death is not related to p53 status and also supports the existence of p53-independent apoptosis in HNSCC cells. Over-expression of Bcl-2 in HNSCC tumour cell lines is unlikely to be a general mechanism to protect these cells from apoptosis. These data support the continuing use of classical apoptosis as an end point for laboratory trials of EGFR inhibitors and cisplatin as the most important outcome in studies of cancer killing is apoptosis.

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REFERENCES

- Al-Hazaa AA, Bowen ID (1998) Improved cytochemical methods for demonstrating cell death using LR White as an embedding medium. *Histochem. J.* **30**, 897.
- Al-Hazaa AA, Bowen ID, Birchall MA (2000) ZD1839 (IressaTM) an EGFR-TKI, and cisplatin have an additive effect on programmed cell death in human head and neck squamous cell carcinoma cells *in vitro*. *Proceedings of the 11th NCI-EORTC-AACR Symposium on New Drugs in Cancer Therapy, Published as a Supplement to Clinical Cancer Research* **6**, ISSN 1078-0432.
- Baselga J (2002) Why the epidermal growth factor receptor? The rationale for cancer therapy. *Oncologist* **7**, 2.
- Campiglio M, Locatelli A, Olgiati C, Normanno N, Somenzi G, Vigano L, Fumagalli M, Menard S, Gianni L (2004) Inhibition of proliferation and induction of apoptosis in breast cancer cells by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor ZD1839 ('Iressa') is independent of EGFR expression level. *J. Cell Physiol.* **198**, 259.
- Caponigro F (2004) Rationale and clinical validation of epidermal growth factor receptor as a target in the treatment of head and neck cancer. *Anticancer Drugs* **15**, 311.
- Chen Z, Ke LD, Yuan XH, Adler-Storthz K (2000) Correlation of cisplatin sensitivity with differential alteration of EGFR expression in head and neck cancer cells. *Anticancer Res.* **20**, 899.
- Choi YJ, Mendoza L, Rha SJ, Sheikh-Hamad D, Baranowska-Daca E, Nguyen V, Smith CW, Nassar G, Suki WN, Truong LD (2001) Role of p53-dependent activation of caspases in chronic obstructive uropathy: evidence from p53 null mutant mice. *J. Am. Soc. Nephrol.* **12**, 983.
- Ciardiello F, Caputo R, Bianco R, Pomato G, Damino V, De Placido S, Bianco AR, Tortora G (1999) Antitumor effects and potentiation of cytotoxic drugs activity in human cancer cell lines by ZD1839 (IressaTM), an EGFR-specific tyrosine kinase inhibitor. *Clin. Cancer Res.* **5**, 28.
- Cohen EE, Rosen F, Stadler WM, Recant W, Stenson K, Huo D, Vokes EE (2003) Phase II trial of ZD1839 in recurrent or metastatic squamous cell carcinoma of the head and neck. *J. Clin. Oncol.* **21**, 1980.
- Daneshmand M, Parolin DA, Hirte HW, Major P, Goss G, Stewart D, Batist G, Miller WH Jr, Matthews S, Seymour L, Lorimer IA (2003) A pharmacodynamic study of the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 in metastatic colorectal cancer patients. *Clin. Cancer Res.* **9**, 2457.
- Di Gennaro E, Barbarino M, Bruzzese F, De Lorenzo S, Pepe S, Caponigro F, Budillon A (1999) Growth inhibition of tumour cells by epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 is associated with up-regulation of p27 and p21 cyclin-dependent kinase inhibitors. *Clin. Cancer Res.* **5**, 118.
- Di Gennaro E, Barbarino M, Bruzzese F, De Lorenzo S, Caraglia M, Abbruzzese A, Avallone A, Comella P, Caponigro F, Pepe S, Budillon A (2003) Critical role of both p27KIP1 and p21CIP1/WAF1 in the antiproliferative effect of ZD1839 ('Iressa'), an epidermal growth factor receptor tyrosine kinase inhibitor, in head and neck squamous carcinoma cells. *J. Cell Physiol.* **195**, 139.

- Fry JC (1993) Biological data analysis. In: Rickwood D, Hames BD, eds. *A Practical Approach*, p. 1. Oxford: IRL Press, Oxford University Press.
- Gonzalez VM, Fuertes MA, Alonso C, Perez JM (2001) Is cisplatin-induced cell death always produced by apoptosis? *Mol. Pharm.* **59**, 657.
- Hague A, Diaz GD, Hicks DJ, Krajewski S, Reed JC, Paraskeva C (1997) bcl-2 and bak may play a pivotal role in sodium butyrate-induced apoptosis in colonic epithelial cells; however overexpression of bcl-2 does not protect against bak-mediated apoptosis. *Int. J. Cancer* **72**, 898.
- Herbst RS (2002) ZD1839: targeting the epidermal growth factor receptor in cancer therapy. *Expert Opin. Invest Drugs* **11**, 837.
- Klijn JG, Berns PM, Schmitz PI, Foekens JA (1992) The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr. Rev.* **13**, 3.
- Magne N, Fischel JL, Dubreuil A, Formento P, Marcie S, Lagrange JL, Milano G (2002) Sequence-dependent effects of ZD1839 ('Iressa') in combination with cytotoxic treatment in human head and neck cancer. *Br. J. Cancer* **86**, 819.
- Magne N, Fischel JL, Tiffon C, Formento P, Dubreuil A, Renee N, Formento JL, Francoal M, Ciccolini J, Etienne MC, Milano G (2003) Molecular mechanisms underlying the interaction between ZD1839 ('Iressa') and cisplatin/5-fluorouracil. *Br. J. Cancer* **89**, 585.
- Mendelsohn J, Baselga J (2000) The EGF receptor family as targets for cancer therapy. *Oncogene* **19**, 6550.
- Min BM, Baek JH, Shin KH, Gujuluva CN, Cherrick HM, Park NH (1994) Inactivation of the p53 gene by either mutation or HPV infection is extremely frequent in human oral squamous cell carcinoma cell lines. *Eur. J. Cancer B Oral Oncol.* **30B**, 338.
- Normanno N, Maiello MR, De Luca A (2003) Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs): simple drugs with a complex mechanism of action? *J. Cell Physiol.* **194**, 13.
- Patel V, Ensley JF, Gutkind JS, Yeudall WA (2000) Induction of apoptosis in head-and-neck squamous carcinoma cells by γ -irradiation and bleomycin is p53-independent. *Int. J. Cancer* **88**, 737.
- Ravi D, Ramadas K, Mathew BS, Panikkar KR, Nair MK, Pillai MR (2001) Apoptosis, angiogenesis and proliferation: trifunctional measure of tumor response to radiotherapy for oral cancer. *Oral Oncol.* **37**, 164.
- Ritter CA, Arteaga CL (2003) The epidermal growth factor receptor-tyrosine kinase: a promising therapeutic target in solid tumors. *Semin. Oncol.* **30**, 3.
- Schiller JH (2003) New directions for ZD1839 in the treatment of solid tumors. *Semin. Oncol.* **30**, 49.
- Shintani S, Li C, Mihara M, Yano J, Terkado N, Nakashiro K, Hamakawa H (2004) Gefitinib ('Iressa'), ZD1839, an epidermal growth factor receptor tyrosine kinase inhibitor, up-regulates p27KIP1 and induces G1 arrest in oral squamous cell carcinoma cell lines. *Oral Oncol.* **40**, 43.
- Sumitomo M, Asano T, Asakuma J, Horiguchi A, Hayakawa M (2004) ZD1839 modulates paclitaxel response in renal cancer by blocking paclitaxel-induced activation of the epidermal growth factor receptor-extracellular signal-regulated kinase pathway. *Clin. Cancer Res.* **10**, 794.
- Vicentini C, Festuccia C, Gravina GL, Angelucci A, Marronaro A, Bologna M (2003) Prostate cancer cell proliferation is strongly reduced by the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 *in vitro* on human cell lines and primary cultures. *Cancer Res. Clin. Oncol.* **129**, 165.
- Zamble DB, Jacks T, Lippard SJ (1998) p53-dependent and -independent responses to cisplatin in mouse testicular teratocarcinoma cells. *Proc. Natl Acad. Sci. USA* **95**, 6163.